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4 **Title**

5 **Enhanced biohydrogen production from corn stover by the combination of**

6 ***Clostridium cellulolyticum* and hydrogen fermentation bacteria**

7 **Running Title: Hydrogen production from corn stover**

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Abstract:

Hydrogen was produced from steam-exploded corn stover by using a combination of the cellulolytic bacterium *Clostridium cellulolyticum* and non-cellulolytic hydrogen-producing bacteria. The highest hydrogen yield of the co-culture system with *Clostridium cellulolyticum* and *Citrobacter amalonaticus* reached 51.9 L H₂/kg TS (Total Solid). The metabolites from the co-culture system were significantly different from those of the mono-culture systems. Formate, which inhibits the growth of *C. cellulolyticum*, could be consumed by the hydrogen-evolving bacteria, and transformed into hydrogen. Glucose and xylose were released from corn stover via hydrolysis by *C. cellulolyticum* and were quickly utilized in dark fermentation with the co-cultured hydrogen-producing bacteria. Because the hydrolysis of corn stover by *C. cellulolyticum* was much slower than the utilization of glucose and xylose by the hydrogen-evolving bacteria, the sugar concentrations were always maintained at low levels, which favored a high hydrogen molar yield.

Key words: mesophilic co-culture; synergic effect; hydrogen production; dark fermentation; corn stover

Introduction

Corn stover is one of the most important cellulosic resources in nature. China produces 0.84 billion tons of corn stover, which are equal to 0.42 billion tons of coal or 11.2% of China's total energy consumption in 2013 (1,2). Many solutions have been proposed to reduce the pollution caused by the waste of corn stover, such as converting corn stover to feed, fuel or industrial ingredients (3). Among these solutions, the conversion of corn stover to gas biofuels is one of the most promising applications (4). For instance, methane production from lignocellulosic waste has been widely investigated (5). In recent years, hythane, a new gas biofuel comprising hydrogen and methane, has been proposed. By adding 10-25% (v/v) hydrogen to methane, the greenhouse gas emissions of methane are reduced, and the fuel efficiency of the composite biofuel is improved, making hythane a promising fuel source (4).

Two-stage dark fermentation is a potential way to produce hythane from lignocellulosic waste (6-8). Hydrogen fermentation can produce hydrogen from corn stover, and the end products, which are mainly organic acids formed in the hydrogen fermentation, could be further utilized by methanogens in methane fermentation (9). Because the methane fermentation process has been widely studied and optimized, the two-stage fermentation of corn stover can be enhanced by improving the hydrogen production rate and the production yield in the hydrogen fermentation process.

Corn stover has a complex composition and structure, making it difficult to degrade and convert. Researchers have focused on mixed-culture microorganisms to

enhance the efficiency of utilization of corn stover and the yield and productivity of hydrogen ([Table 1](#)). When anaerobic sludge was used as the microbial consortium, the hydrogen production yield reached between 45.6 and 64.6 L/kg TS ([10,12,13](#)). However, this natural consortium is inefficient and difficult to control and thereby poorly optimized ([4,9](#)). A more effective strategy to improve the hydrogen yield is bioaugmentation. By adding cellulolytic microorganisms to the microbial consortium, the hydrolysis of corn stover and the hydrogen yield were further improved. *Clostridium thermocellum* was added to anaerobic sludge to enhance hydrogen production from corn stover, resulting in a maximum yield of 63.7 L/kg TS ([9](#)). However, the effects of bioaugmentation are limited if the introduced exogenous microorganism cannot symbiotically survive with the other members of the consortium ([9](#)). To more efficiently produce hydrogen from corn stover, an attractive strategy would be to engineer an artificial consortium capable of performing biomass saccharification and hydrogen fermentation. For instance, the co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* was developed to improve hydrogen production via the thermophilic fermentation of cornstalk waste at maximum hydrogen yields of 68.2 L/kg TS ([14](#)) and 105.6 L/kg TS (with dynamic microwave-assisted alkali pretreatment) ([15](#)). However, these yields were produced under thermophilic conditions; most biochemical reactions naturally occur under mesophilic conditions. Furthermore, mesophilic fermentation requires less energy and has better process stability ([16](#)). Therefore, it is necessary to construct an artificial consortium capable of producing hydrogen from corn stover under mesophilic

conditions. The aims of this study were to combine a cellulolytic bacterium with different hydrogen-producing bacteria and to evaluate the biohydrogen production of each combination by mesophilic fermentation from steam-exploded corn stover. Furthermore, this study investigated the synergic effect and mechanism of each combination.

Clostridium cellulolyticum, a mesophilic, Gram-positive microorganism isolated from decayed grass compost, possesses cellulolytic activity through cellulosomes (17-20) and was used as a model cellulolytic bacterium. *Clostridium paraputrificum*, *Citrobacter amalonaticus*, *Enterobacter cloacae* and *Enterobacter aerogenes* were used as the hydrogen fermentation bacteria. These four hydrogen fermentation strains exhibited relatively high hydrogen yields or high hydrogen production rates and have been used for co-culture (21-25). The hydrogen production and synergic effect between each combination were compared, and the effects of formate on the co-culture systems were investigated. This work also found that the release rates of sugars hydrolyzed from corn stover were much slower than the utilization rates of sugars consumed by the hydrogen-producing strains and could contribute to the high hydrogen yields from corn stover.

Materials and methods

Materials and bacterial strains

Corn stover was harvested from a rural area near Beijing and pretreated by steam explosion as previously described (26).

The hydrogen-producing strains of *Enterobacter aerogenes* IAM1183 and *Clostridium paraputrificum* M-21 were previously described by Lu et al. (9) The *Enterobacter cloacae* (NBRC 12935) and *Citrobacter amalonaticus* Y19 (NBRC 13547) strains were purchased from NBRC, and the cellulolytic strain of *Clostridium cellulolyticum* (DSM 5812) was purchased from DSMZ.

Medium and cultivation

The preculture medium (1000 mL) for *Clostridium cellulolyticum* consisted of 6.0 g of cellobiose, 2.0 g of yeast extract, 1.3 g of $(\text{NH}_4)_2\text{SO}_4$, 1.5 g of KH_2PO_4 , 2.9 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, of CaCl_2 0.075 g, 1.25 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mg of resazurin, 1.0 mL of Trace Elements Solution and 0.5 g of cystine-HCl-H₂O. The Trace Elements Solution (1000 mL) consisted of 10 mL of HCl (25%, 7.7 M), 1.50 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 70 mg of ZnCl_2 , 100 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6 mg of H_3BO_3 , 190 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mg of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 36 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 990 mL of distilled water. The preculture medium (1000 mL) for the hydrogen-producing bacteria consists of peptone 10 g, yeast extract 2 g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g. The medium used for hydrogen fermentation was the same as that for *Clostridium cellulolyticum* except for the carbon sources.

The hydrogen fermentation process was conducted in 70-mL serum bottles containing 20 mL of medium. The inoculum size for each bottle was 5% (v/v). All bottles were airtight and fitted with butyl rubber stoppers. Nitrogen (99.999%) was bubbled through the medium for 25 min prior to distributing the medium into the serum bottles in an anaerobic chamber. The cultivation was performed at 37 °C. After

a 121 °C, 20 min autoclave treatment, the medium pH value was adjusted to 7.2 using 1 M NaOH.

Analytical Methods

The hydrogen content in the gas phase of the cultures was analyzed with a gas chromatography (GC) instrument (GC112A, Shanghai Precision and Scientific Instrument Co., Ltd.) equipped with a thermal conductivity detector (TCD) and a 2 m × 3 mm (i.d.) stainless-steel column packed with TDX-01 (80–100 mesh). The concentrations of end metabolites in the liquid phases of the cultures were analyzed by a high performance liquid chromatography (HPLC) system (Shimadzu 10A) equipped with an RID detector and a Shodex RSpac KC-811 column. The mobile phase was 0.1% (w/v) HClO₄. Detailed parameters of the GC and HPLC analyses were the same as previously described (9).

The concentrations of soluble sugars were measured by a phenol-sulfuric acid method (27). Holocellulose content was determined by decreases in dry weight.

Results and discussion

Hydrogen production from corn stover by co-culture fermentation

The strains used in this study include cellulolytic and non-cellulolytic mesophilic strains (Table 2). *Clostridium cellulolyticum* was used as the cellulolytic bacterium. *Clostridium paraputrificum*, *Citrobacter amalonaticus*, *Enterobacter cloacae* and *Enterobacter aerogenes* were used as the hydrogen fermentation bacteria. The optimum temperatures and pH ranges of the different strains were similar. The

cellulolytic and non-cellulolytic hydrogen-evolving bacteria were paired to compare their hydrogen production efficiencies with 25 g/L steam-exploded corn stover as the substrate.

As shown in [Fig. 1](#), the control monoculture of the non-cellulolytic hydrogen-evolving bacterium accumulated small amounts of hydrogen due to 12% (w/w) soluble sugars present in steam-exploded corn stover. The monoculture of the cellulolytic bacterium *C. cellulolyticum* did not accumulate H₂. The combination co-culture of the cellulolytic and non-cellulolytic bacteria showed enhanced H₂ accumulation to more than 330 mL H₂/L medium after a four-day cultivation. In particular, the combination of *C. amalonaticus* Y19 and *C. cellulolyticum* showed the highest production at 467±28 mL H₂/L medium, which corresponds to 51.9 L/kg TS. To the best of our knowledge, this was the first artificially constructed mesophilic co-culture system for producing hydrogen from corn stover. When compared with the studies in [Table 1](#), this result was nearly equivalent to the hydrogen production yields by anaerobic sludge and monocultures under thermophilic conditions [\(10,11,13\)](#). However, the co-culture fermentation cultures under thermophilic conditions had higher hydrogen yields [\(14,15\)](#). This is primarily due to the strong influence of temperature on the activity of the microorganisms and the yield of the target product [\(16\)](#).

[Fig. 2](#) shows variations in hydrogen production and culture growth of four hydrogen-producing strains (with 6 g/L glucose and 25 g/L corn stover as the sole carbon source). When using glucose as the sole carbon source, the growth

of these four strains exhibited similar trends as those observed for hydrogen production: the OD₆₀₀ and hydrogen volume rapidly increased in the first 5 h of cultivation and gradually stabilized after 8 h. However, when using corn stover as the sole carbon source, although the hydrogen volume increased continuously during the fermentation, the OD₆₀₀ started to decrease after 64 h of cultivation. This result indicated that hydrogen-producing bacteria could not produce hydrogen when the sugars in the system were used up. The co-culture system could supply sugars and thereby, improve hydrogen production.

Xylose is one of the most important components in corn stover. However, the effective utilization of both glucose and xylose has posed a problem in the fermentative production of other biofuels, such as bioethanol. Mohand-Oussaid et al. (28) studied the extracellular xylandegradative system in *Clostridium cellulolyticum* and confirmed that *C. cellulolyticum* could degrade both cellulose and hemicelluloses and consequently, release both glucose and xylose. In this study, all hydrogen-producing strains used could use both glucose and xylose as the sole carbon source to produce hydrogen. When the carbon source was a 9 g/L mixed sugar medium with equal concentrations of glucose, xylose and cellobiose, all strains preferentially consumed glucose. Interestingly, in the *C. cellulolyticum* and *C. paraputrificum* co-culture, xylose was also consumed even in the presence of glucose. These results indicated that the combinations of *C. cellulolyticum* with hydrogen-producing strains could simultaneously metabolize glucose and xylose released from corn stover to produce hydrogen, which could

contribute toward high hydrogen yields from corn stover.

Comparison and analysis of metabolites in the monoculture and co-culture systems

Metabolites from the co-cultures of *C. cellulolyticum* with hydrogen fermentation bacteria and those of the *C. cellulolyticum* monoculture were compared. As shown in Table 3, the end products of the *C. cellulolyticum* monoculture were primarily acetate, formate, ethanol, lactate, and 2,3-butanediol, while those of the hydrogen producing strains were primarily acetate and ethanol and, to a lesser extent, some succinate, lactate and 2,3-butanediol. Interestingly, formate was not detected in the end products of the co-culture systems but was one of the main products of the *C. cellulolyticum* monoculture. This difference indicated that formate, as the end metabolic product of *C. cellulolyticum*, could be metabolized by the hydrogen fermentative bacteria. Experiments were conducted to show that the hydrogen fermentative strains used in this research could produce hydrogen using different concentrations of formate (0 mM, 5 mM, 10 mM and 20 mM) as the sole carbon source (Fig. 3). The results indicated that the formate produced by the *C. cellulolyticum* was quickly metabolized by the hydrogen producing bacteria in the co-culture systems. Furthermore, the influence of formate accumulation on *C. cellulolyticum* was studied by adding different concentrations of formate (0 mM, 5 mM and 10 mM) to the culture media. As shown in Fig. 4, the growth of *C. cellulolyticum* was obviously inhibited with the additional formate. Therefore, formate, which was produced by *C. cellulolyticum*, could inhibit the growth of *C.*

cellulolyticum. By combining with hydrogen fermentation bacteria, the accumulated formate could be removed and further transformed into hydrogen.

Another interesting aspect was the ethanol/acetate ratios of the different systems varied. The ethanol/acetate ratio is an index of the oxidation-reduction potential (ORP), which is highly correlated with hydrogen production. A higher ethanol/acetate ratio indicates a more reductive environment, which is favorable for hydrogen metabolism (29). Table 3 shows that the co-culture fermentations had higher ethanol/acetate ratios than the monoculture hydrogen fermentation, indicating that the combinations of hydrogen fermentative strains, especially facultative anaerobic bacteria, with *C. cellulolyticum* could decrease the ORP and provide a more favorable environment for hydrogen production.

Comparing the hydrogen yields of the monoculture and co-culture system

The hydrogen yields of the *C. paraputrificum* co-culture with *C. cellulolyticum* (with 10 g/L corn stover as substrate) and the monoculture of *C. paraputrificum* (with 10 g/L glucose as substrate) were compared (Table 4). While the hydrogen produced by the co-culture system was less than half that of the monoculture, the molar yield of hydrogen was much higher (1.97 mol H₂/mol hexose) in the co-culture system.

To explain this phenomenon, hydrogen production by four different strains for different concentrations of glucose (5 mM, 10 mM, 25 mM, 50 mM, 75 mM and 125 mM) were compared. As shown in Fig. 5, for all hydrogen-producing strains, the hydrogen molar yields were much higher at lower glucose concentrations. These results were identical with those reported by a previous study wherein high glucose

concentrations lowered the hydrogen molar yield when *C. amalonaticus* Y19 was used as inoculum (23). It is clear that the initial glucose concentration is an important factor that significantly influences hydrogen fermentation. In the co-culture system, hydrogen fermentation could also be affected by the inoculum proportion. Fig. 6 shows the results of hydrogen production by the co-culture of *C. paraputrificum* and *C. cellulolyticum* with different inoculum proportions (with 10 g/L corn stover as substrate). When the proportion of *C. cellulolyticum* increased, the volume of hydrogen also increased. In this case, the hydrolysis of corn stover was the rate-limiting step of the fermentation. The higher numbers of *C. cellulolyticum* increased the amount of sugars available from the corn stover and stabilized the co-culture system. However, the growth of hydrogen was only 50% when the amount of *C. cellulolyticum* doubled. This result was consistent with the results shown in Fig. 5. Because the release rate of sugars hydrolyzed from corn stover was much slower than the utilization rate of sugars consumed by the hydrogen-producing strains, the sugar concentration was always maintained at a low level, which could be one of the reasons for the higher hydrogen molar yield of corn stover in the co-culture system compared with that of the monoculture of the hydrogen-producing strain using the glucose-containing medium mentioned above.

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Figure legends

Fig. 1 Hydrogen production by different co-culture systems from corn stover after 96 h cultivation. Error bars represent standard deviations from triplicate cultures. Diagonal: raw gas; blank: hydrogen. 1: *C. cellulolyticum*; 2: *C. paraputrificum*; 3: *C. amalonaticus*; 4: *E. cloacae*; 5: *E. aerogenes*; 6: *C. cellulolyticum* and *C. paraputrificum*; 7: *C. cellulolyticum* and *C. amalonaticus*; 8: *C. cellulolyticum* and *E. cloacae*; 9: *C. cellulolyticum* and *E. aerogenes*.

Fig. 2 The growth and hydrogen production changes in the individual culture of different strains. (A) time-course profiles of hydrogen production by using glucose as the carbon source; (B) time-course profiles of OD₆₀₀ by using glucose as the carbon source; (C) time-course profiles of hydrogen production by using corn stover as the carbon source; (D) time-course profiles of OD₆₀₀ by using corn stover as the carbon source. Error bars represent standard deviations from triplicate cultures. Square: *C. paraputrificum*; circle: *C. amalonaticus*; normal triangle: *E. cloacae*; inverted triangle: *E. aerogenes*.

Fig. 3 Hydrogen production from formate at different initial concentrations by different hydrogen producers. Error bars represent standard deviations from triplicate cultures. Square: *C. paraputrificum*; circle: *C. amalonaticus*; triangle: *E. cloacae*.

Fig. 4 Influence of formate addition on the growth of *C. cellulolyticum*. Dark symbols: pH; white symbols: OD₆₀₀. Square: 0 mM formate; triangle: 5 mM formate; circle: 10 mM formate.

Fig. 5 Influence of initial glucose concentrations on hydrogen mole yields by the different hydrogen producers. Error bars represent standard deviations from triplicate cultures. Square: *C. amalonaticus*; circle: *C. paraputrificum*; normal triangle: *E. cloacae*; inverted triangle: *E. aerogenes*.

Fig. 6 Influence of inoculum proportion on hydrogen production by the co-culture of *C. cellulolyticum* and *C. paraputrificum*. Error bars represent standard deviations from triplicate cultures. Dark symbols: raw gas; white symbols: hydrogen. Square: *C. cellulolyticum* : *C. paraputrificum* = 1:1; circle: *C. cellulolyticum* : *C. paraputrificum* = 2:1.

Fig. 1 Hydrogen production by different co-culture systems from corn stover after 96 h cultivation. Error bars represent standard deviations from triplicate cultures.

Fig. 2 The growth and hydrogen production changes in the individual culture of different strains. (A) time-course profiles of hydrogen production by using glucose as the carbon source; (B) time-course profiles of OD₆₀₀ by using glucose as the carbon source; (C) time-course profiles of hydrogen production by using corn stover as the carbon source; (D) time-course profiles of OD₆₀₀ by using corn stover as the carbon source. Error bars represent standard deviations from triplicate cultures.

Fig. 3 Hydrogen production from formate at different initial concentrations by different hydrogen producers. Error bars represent standard deviations from triplicate cultures.

Fig. 4 Influence of formate addition on the growth of *C. cellulolyticum*

Fig. 5 Influence of initial glucose concentrations on hydrogen mole yields by the different hydrogen producers. Error bars represent standard deviations from triplicate cultures.

Fig. 6 Influence of inoculum proportion on hydrogen production by the co-culture of *C. cellulolyticum* and *C. paraputrificum*. Error bars represent standard deviations from triplicate cultures.

Table 1 Previous studies about hydrogen production from corn stover

Inoculum	Pretreatment	Operation	H ₂ Yield (L/kg TS)	Reference
Heat-treated sludge and <i>Clostridium thermocellum</i>	Steam-explosion	Serum bottle, batch, 55 °C	63.7	(9)
Anaerobic sludge <i>Thermoanaerobacterium</i> <i>thermosaccharolyticum</i>	microwave-assisted acid	Serum bottle, batch, 55 °C	45.6	(10)
W16	Alkali and enzymatic hydrolysis	Flakes, batch, 60 °C	44.5	(11)
Activated sludge and anaerobic granular sludge	Acid hydrolysis	Serum vials, batch, 30-70 °C	64.6 (55 °C)	(12)
Anaerobic sludge <i>Clostridium thermocellum</i>	Fungal pretreatment	Serum bottle, batch, 55 °C	48.7	(13)
and <i>Clostridium</i> <i>thermosaccharolyticum</i>	Mill to powder	Anaerobic bottle, batch, 55 °C	68.2	(14)
<i>Clostridium thermocellum</i> and <i>Clostridium</i> <i>thermosaccharolyticum</i>	Dynamic microwave-assisted alkali	Anaerobic bottle, batch, 55 °C	105.6	(15)

Note: The value of Each H₂ yield was converted into a single unit.

Table 2 Properties of the strains used in this study

Strains	Optimum T / °C	Optimum pH	Function	Feature
<i>Clostridium cellulolyticum</i> ATCC 35319	35~37	6.8~7.2	Cellulose degradation	Obligate anaerobic
<i>Citrobacter amalonaticus</i> Y19	37	7.0	Hydrogen fermentation	Facultative anaerobic
<i>Clostridium paraputrificum</i> M21	37	7.0	Hydrogen fermentation	Obligate anaerobic
<i>Enterobacter cloacae</i> IIT-BT 08	32~40	5~6	Hydrogen fermentation	Facultative anaerobic
<i>Enterobacter aerogenes</i> IAM1183	37	7.0	Hydrogen fermentation	Facultative anaerobic

Table 3 Comparisons of end products in different monoculture and co-culture fermentation systems.

	Metabolites / mM						Ethanol/ Acetate
	Succinate	Lactate	Formate	Acetate	2,3-butanediol	Ethanol	
<i>C. cellulolyticum</i>	--	0.34±0.12	5.51±1.47	6.01±1.80	0.18±0.02	2.36±0.80	0.39
<i>C. paraputrificum</i>	0.37±0.07	0.91±0.93	--	3.84±0.68	0.94 ^a	0.94±0.15	0.24
<i>C. cellulolyticum</i> and <i>C. paraputrificum</i>	0.46±0.03	0.91 ^a	--	6.32±0.24	--	1.78±0.12	0.28
<i>C. amalonaticus</i>	0.29±0.04	1.02±0.82	--	5.00±0.44	--	0.37±0.12	0.07
<i>C. cellulolyticum</i> and <i>C. amalonaticus</i>	0.28±0.03	0.31±0.02	--	6.82±0.53	--	1.84±0.34	0.27
<i>E. cloacae</i>	0.27 ^a	1.32±0.93	--	1.16±0.36	0.21±0.04	0.23 ^a	0.20
<i>C. cellulolyticum</i> and <i>E. cloacae</i>	0.24±0.04	1.48 ^a	--	4.04±0.52	0.16±0.03	1.80±0.18	0.45
<i>E. aerogenes</i>	0.01±0.01	0.50±0.28	--	1.23±0.40	0.01±0.00	0.09±0.04	0.07
<i>C. cellulolyticum</i> and <i>E. aerogenes</i>	0.65±0.05	0.60 ^a	--	5.78±0.56	0.41 ^a	2.36±0.24	0.41

Note: -- undetectable; ^a single sample; all of the experiments were conducted in triplicate and all values were the average values (except for the values marked by letters) of triplicate ± standard deviations.

Table 4 Comparison of the hydrogen molar yields by co-culture from corn stover and monoculture from glucose.

	Carbon source	Hydrogen yield	Mole yield
	10 g/L	L/kg TS	mol/mol glucose
<i>C. cellulolyticum</i> and <i>C. paraputrificum</i> ^a	Corn stover	40.3±4.2	1.97±0.07
<i>C. paraputrificum</i> ^b	Glucose	92.0±10.9	0.77±0.09

Note: *a* 96 h cultivation; *b* 24 h cultivation; all of the experiments were conducted in triplicate and all values were the average values of triplicate ± standard deviations.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6