Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2016

www.elsevier.com/locate/jbiosc

Enhanced biohydrogen production from corn stover by the combination of *Clostridium cellulolyticum* and hydrogen fermentation bacteria

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Received 6 November 2015; accepted 18 March 2016
Available online xxx

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 *C. cellulolyticum* and *Citrobacter amalonaticus* reached 51.9 L H_2/kg total solid (TS). 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]

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 single strain or mixed-culture microorganisms to enhance the efficiency of utilization of corn stover and the yield and productivity of hydrogen (Table 1) (9–15). When anaerobic sludge was used as the microbial consortium, the hydrogen production yield reached between 45.6 and 64.6 L/kg total solid (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 *C. 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

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.03.014

TABLE 1. Previous studies about hydrogen production from corn stover.

Inoculum	Pretreatment	Operation	H_2 yield (L/kg TS)	Reference
Heat-treated sludge and *Clostridium thermocellum*	Steam-explosion	Serum bottle, batch, 55°C	63.7	9
Anaerobic sludge	microwave-assisted acid	Serum bottle, batch, 55°C	45.6	10
Thermoanaerobacterium thermosaccharolyticum 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	Fungal pretreatment	Serum bottle, batch, 55°C	48.7	13
Clostridium thermocellum and *C. thermosaccharolyticum*	Mill to powder	Anaerobic bottle, batch, 55°C	68.2	14
Clostridium thermocellum and *C. thermosaccharolyticum*	Dynamic microwave-assisted alkali	Anaerobic bottle, batch, 55°C	105.6	15

Note: The value of each H_2 yield was converted into a single unit.

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

Medium and cultivation The preculture medium (1000 mL) for *C. cellulolyticum* consisted of 6.0 g of cellobiose, 2.0 g of yeast extract, 1.3 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 2.9 g of $K_2HPO_4 \cdot 3H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, of $CaCl_2$ 0.075 g, 1.25 mg of $FeSO_4 \cdot 7H_2O$, 1.0 mg of resazurin, 1.0 mL of Trace Elements Solution and 0.5 g of cystine-HCl·H_2O. The trace elements solution (1000 mL) consisted of 10 mL of HCl (25%, 7.7 M), 1.50 g of $FeCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 100 mg of $MnCl_2 \cdot 4H_2O$, 6 mg of H_3BO_3, 190 mg of $CoCl_2 \cdot 6H_2O$, 2 mg of $CuCl_2 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, 36 mg of $Na_2MoO_4 \cdot 2H_2O$ 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 $MgSO_4 \cdot 7H_2O$ 1 g. The medium used for hydrogen fermentation was the same as that for *C. 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) $HClO_4$. 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). *C. cellulolyticum* was used as the cellulolytic bacterium. *C. paraputrificum*, *C. amalonaticus*, *E. cloacae* and *E. 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 H_2. The combination co-culture of the cellulolytic and non-cellulolytic bacteria showed enhanced H_2 accumulation to more than 330 mL H_2/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 H_2/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 OD_{600} and hydrogen volume rapidly increased in the first 5 h of cultivation and gradually stabilized after 8 h. However, when using

TABLE 2. Properties of the strains used in this study.

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

Please cite this article in press as: Zhang, S.-C., et al., Enhanced biohydrogen production from corn stover by the combination of *Clostridium cellulolyticum* and hydrogen fermentation bacteria, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.03.014