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Bioaugmented hydrogen production from carboxymethyl cellulose and partially delignified corn stalks using isolated cultures

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ABSTRACT

Dark fermentation of carboxymethyl cellulose to produce biohydrogen using mono-culture or co-culture of isolated strains was studied. Three isolates were noted to effectively hydrolyze cellulosic substrates and degrade the metabolites to hydrogen and volatile fatty acids. The strain *Clostridium acetobutylicum* X₉ was noted to have the highest hydrogen yield amongst the three isolates in all tests. Co-cultures of any two of the three isolates and with another strain *Ethanoigenens harbinense* B₄₉ demonstrated higher biohydrogen yield and cellulose hydrolysis ratio compared with the mono-cultured tests. Bioaugmentation with co-cultures X₉ + B₄₉ efficiently improved cellulose hydrolysis and subsequent hydrogen production rates from carboxymethyl cellulose. The strain X₉ significantly hydrolyzed corn stalks pretreated with H₂SO₄, NaOH, and NH₃ soaking, and steam explosion in 10–12 h. Hydrogen was yielded in conjunction with the noted cellulose hydrolysis. The steam explosion + hydrolysis/fermentation with X₉ present the most effective method amongst the four tested pretreatments for hydrolyzing cellulose and yielding hydrogen.

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1. Introduction

Anaerobic fermentation processes convert biomass to hydrogen [1–4]. Production of biohydrogen from waste materials is considered as an attractive future energy source [5–9]. Cellulose is a predominant constituent of agricultural waste and waste generated by the pulp and paper industry. Lay [10] investigated the potential of producing hydrogen from microcrystalline cellulose using mesophilic digestion with heat-shocked sludge. Liu et al. [11] determined that their mixed culture comprising microbes closely affiliated with the genus *Thermoanaerobacterium* produced hydrogen that

peaked at 7.56 mg H₂ g^{−1} cellulose from a 5 g cellulose l^{−1} suspension maintained at pH 6.5 and 55 °C. Wang et al. [12] reported enhanced biohydrogen production from microcrystalline cellulose using two cultures, in batch reactor.

To generate hydrogen directly from lignocellulose materials using dark fermentation normally requires expensive pretreatment processes such as delignification and hydrolysis to dissolve organic matter from a lignocellulose complex [13–15]. For instance, steam explosion treatment forces high pressure steam to penetrate the lignocellulosic structures to hydrolyze the hemicellulose fractions and releases suddenly the pressure to breakdown lignocellulosic structures. Acid

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treatment uses hydrogen ions as catalyst to hydrolyze the glycosidic bonds at the surfaces or in the amorphous regions of the cellulose. Pretreatment processes are in general expensive and time consuming. Mesophilic isolates (bacteria) that effectively hydrolyze the partially delignified lignocellulose complex are of significant practical interest to decrease biohydrogen production cost.

This study isolated nine strains from a full-scale hydrogen fermenter. The strains that could effectively produce biohydrogen from microcrystalline cellulose or carboxymethyl cellulose suspensions were tested. The isolated strain, X₉, was used to hydrolyze and produce hydrogen from pretreated corn stalks.

2. Materials and methods

2.1. Strain cultivation

Liquor samples were obtained from a pilot-scale hydrogen fermenter in Harbin, China, which is a continuous flow anaerobic fermentation reactor with working volume of 1.48 m³ and fed with molasses as the substrate. The operation was performed under organic loading rates of 3.11–85.8 kg COD m⁻³ d⁻¹ for over 200 days. The liquid samples were placed in aseptically mixed sterilized tubes containing 50 ml medium containing cellulosic materials. The compositions are (per liter): microcrystalline cellulose or CMC 10 g, peptone 4.0 g, beef extract 2.0 g, yeast extract 1.0 g, MgCl₂ 0.15 g, FeSO₄ 0.15 g, NaCl 1.0 g,

K₂HPO₄ 1.5 g, and L-cysteine 0.5 g. Moreover, 10 ml l⁻¹ medium of both vitamins (per liter: cyanocobalamin 0.01 g, ascorbic acid 0.025 g, riboflavin 0.025 g, citric acid 0.02 g, pyridoxin 0.05 g, folic acid 0.01 g, 4-aminobenzoic acid 0.01 g, and creatine 0.025 g) and micronutrients (MnSO₄·7H₂O 0.01 g, ZnSO₄·7H₂O 0.05 g, H₃BO₃ 0.01 g, N(CH₂COOH)₃ 0.01 g, CaCl₂·2H₂O 0.01 g, Na₂MoO₄ 0.01 g, CoCl₂·6H₂O 0.2 g, AlK(SO₄)₂ 0.01 g), and 1 ml of resazurin (0.2%) were also added.

The mixtures were incubated at 37 °C, anaerobic condition for 18 h. Suspension samples collected from the incubated tubes were plated on agar plates. Nine isolates from screened strains were tested for their potential to produce hydrogen from microcrystalline cellulose or CMC.

2.2. Fermenting test – CMC

Fermentation tests were performed using the same medium as that in cultivation stage (Section 2.1). Briefly, 50 ml sterilized medium was mixed with 10 ml inoculum (biomass of 0.375 g dry cells) and was kept at 37 °C, anaerobic condition for 18 h. The strain that had highest hydrogen potential among those isolates from microcrystalline cellulose or CMC suspensions was further tested.

Another strain isolated by authors in Ref. [15], B₄₉, AF481148 in NCBI, was utilized to co-work with the isolated strain X₉ in fermentation tests. In the co-culture tests, the two strains were mixed at the same volumes at fixed total biomass quantities (0.375 g dry cells).

Table 1 – Batch fermentation tests of nine isolates and their co-cultures with microcrystalline cellulose or CMC as substrates

Substrate	Strain	H ₂ ^a (ml)	pH	Y _{H₂} (ml l ⁻¹)	Metabolite (mg l ⁻¹)		
					Ethanol	Acetate	Butyrate
Microcrystalline cellulose	C ₃	13.4	4.32	268	1120	982	0
	D ₁	0	6.05	0	91	160	0
	BB	0	5.66	0	252	274	0
	BC	0	5.87	0	229	255	0
	2B	0	4.84	0	222	255	0
	B ₂	3.5	4.45	71	801	740	0
	X ₉	33.2	4.21	664	0	2136	2353
	B ₄₉ + B ₂	100	3.93	940	725	426	0
	B ₄₉ + C ₃	87	4.04	737	691	423	0
	B ₂ + C ₃	89	3.95	864	1030	716	0
	X ₉ + B ₄₉	127	3.79	1810	1780	2060	987
	X ₂ + B ₂	131	3.82	1680	2780	2120	521
	X ₉ + C ₃	102	3.63	1290	2060	1660	1330
	C ₃	12.5	4.43	249	1079	938	0
	D ₁	0	5.79	0	92	139	0
Carboxymethyl cellulose	BB	0	5.77	0	291	281	0
	BC	0	5.94	0	216	225	0
	2B	0	5.87	0	202	228	0
	B ₂	3.3	4.47	66	768	787	0
	X ₉	30	4.23	602	0	2006	2200
	B ₄₉ + B ₂	91	4.16	792	1080	683	0
	B ₄₉ + C ₃	84	4.18	702	926	531	0
	B ₂ + C ₃	110	4.09	932	1150	780	0
	X ₉ + B ₄₉	130	3.87	1750	2510	2110	1040
	X ₂ + B ₂	102	3.93	1450	1580	1320	1590
	X ₉ + C ₃	110	3.85	1450	1850	1690	1720

a Hydrogen production owing to reduced sugar in incubation medium was deduced.

Fig. 1 – The magnified image of strain X_9 , 400 $\times$.

2.3. Tests with corn stalks

The corn stalks used in this study was taken from the agricultural area in countryside close to Harbin city.

Four pretreatment methods were adopted to pretreat the corn stalks, including the use of H_2SO_4 (0.2, 0.5, 1% w/v), NaOH (0.5, 1.0, 2.0% w/v), $NH_3 \cdot H_2O$ (2.0, 5.0, 10% w/v), and steam explosion with H_2SO_4 . In the former three chemical treatments, 15 g dried corn stalks powder (30–60 meshes) was mixed at room temperature with the solutions at prescribed concentrations at solid: solution ratio of 1:10 (w/w) for 24 h. In the steam explosion treatment, 15 g dried corn stalks powder (30–60 meshes) was first mixed with 150 ml, 1% w/v H_2SO_4 and was placed in an autoclave kept at 121 $^{\circ}C$, 15 psi for 2 h. The residue following steam explosion was filtered with water to separate it into lignocellulose solid (LS) and hydrolyzed solution (HS). The LS fraction was tested with X_9 .

In fermentative tests, hydrogen production capabilities, liquid end products, cellulose degradation rate, quantities of reductive saccharides, and suspension pH were measured.

2.4. Analytical methods

A wet gas meter was utilized to measure biogas production volume at room temperature. A gas chromatography (GC112, Perkin-Elmer, USA) with nitrogen as the carrier gas was used to determine VFA and alcohols content in centrifugated (4000 rpm) fermenting liquor. The hydrogen flame detector with hydrogen and air flow rates of 50 and 490 $ml\ min^{-1}$, respectively, and a stainless steel column (2 m) packed with GDX-103 (60–80 meshes) were employed at 190 $^{\circ}C$. Hydrogen content in the gas phase was determined by gas chromatography with nitrogen as the carrier gas (70 $ml\ min^{-1}$) and a thermal conductivity detector. The column (2 m) was packed with TDS-01 (60–80 meshes). The column and detector were kept at 150 $^{\circ}C$. Based on the measured hydrogen production quantity and cell amount, the maximum hydrogen production rate was calculated as $Q_{H_2} = (\text{maximum hydrogen production rate}) \times (\text{hydrogen content in gas}) / \text{dry cell mass}$ ($mmol\ g^{-1}\ dry\ cells$). The specific hydrogen yield (Y_{H_2}) was approximated as $Y_{H_2} = (\text{accumulated biogas production quantity}) \times (\text{hydrogen content in gas}) / (\text{medium volume})$.

The hydrolysis content in cellulosic materials was estimated using the carbon balance calculation as follows [12]:

$$\text{Hydrolysis ratio (\%)} = (C(CO_2) + C(EtOH) + C(Ac) + C(Bu) + C(\text{reduced sugar})) / C(\text{cellulose})$$

A scanning electron microscope (model S-4500, Hitachi Co., Japan) was utilized to assess the morphology of isolates.

A control test conducted with all applied nutrients but no cellulosic materials, which revealed minor hydrogen production compared with the ones with added cellulose. Hence, the effects of added extracts and peptone on hydrogen production are not discussed herein.

3. Results and discussion

3.1. Isolated strains and screening tests

All nine strains were noted capable of hydrolyzing microcrystalline cellulose and CMC to fatty acids. However, only three of them, X_9 , B_2 , and C_3 , had high hydrogen yields from both

Fig. 2 – Biogas production quantity and hydrogen yield (a) and cellulose hydrolysis ratio (b) for co-cultured strains X_9 + B_{49} at 37 $^{\circ}C$ and pH 7.0. CMC content 25 $g\ l^{-1}$ medium; 50 ml medium. Total cell dry mass 0.375 g.

Fig. 3 – Time course of suspension pH in mono-cultured and co-cultured tests. Experimental conditions corresponding to those in Fig. 2.

cellulosic materials (Table 1). The associated metabolites for strain X_9 were primarily acetate and butyrate; whereas those for B_2 and C_3 were ethanol and acetate. That is, strain X_9 had typical butyrate-type fermentation metabolism [17], whereas B_2 and C_3 underwent so-called ethanol-type fermentation metabolism. The strain X_9 has 96% sequence similarity with *Clostridium acetobutylicum* ATCC 824 [12].

At 37 °C and pH 5.0, the co-cultures of any two of the three strains, X_9 , B_2 and C_3 , exhibited 3–4 times hydrogen yields compared with the mono-culture tests. Particularly, the co-cultures of X_9 and strain *Ethanoigenens harbinense* B_{49} , which are a gram-positive, strict anaerobe that cannot utilize cellulosic materials [16], produced the highest hydrogen yield from both cellulosic materials amongst all strain pairs. Meanwhile, the strain X_9 yielded the highest quantities of volatile fatty acids from the two cellulosic materials. To determine the maximum hydrogen yield the present isolates could

derive from CMC, strain X_9 is discussed further in subsequent sections (Fig. 1).

3.2. Mono-culture X_9 and co-cultured $X_9 + B_{49}$ tests using CMC

Fig. 2 presents the hydrogen yield and cellulose hydrolysis ratio for strain X_9 to degrade CMC at 37 °C and pH 5.0. With no lag time, biogas was yielded (Fig. 2a) together with the noted cellulose hydrolysis (Fig. 2b). However, hydrogen was present only on 3 h and above. Hydrogen production and cellulose hydrolysis were completed in 5 h. About 52% of CMC was hydrolyzed by X_9 . The strain X_9 has effectively hydrolyzed CMC and then converted it to hydrogen.

The co-culture $X_9 + B_{49}$ completed fermentation process in 5 h, generating a high cellulose hydrolysis ratio of 70%. The hydrogen yield by strains $X_9 + B_{49}$ was roughly 2.7 times that by the mono-culture test with only X_9 (Fig. 2a).

In a mono-cultured test with X_9 , the suspension pH dropped from 6.7 during the initial 3 h to approximately 4.2, as shown in Fig. 3. In this initial stage, only 10% of CMC was hydrolyzed. The subsequent cellulose hydrolysis and hydrogen formation did not change further the suspension pH. The end liquid products of mono-culture test were primarily acetate and butyrate (Fig. 4a), whose molar ratio (acetate/butyrate) was near 1.5.

In the co-culture test with $X_9 + B_{49}$, the pH dropped to near 4.1 in merely 1 h of test (Fig. 3). The end liquid products were acetate, ethanol, and butyrate (Fig. 4b), with a molar ratio (acetate/ethanol/butyrate) of nearly 2:1:1. That strain B_{49} degrades glucose via an ethanol-type fermentation pathway [17]. The co-culture could rapidly hydrolyze cellulose and produce hydrogen, indicating the presence of certain bioaugmentation between these two strains on cellulose hydrolysis/hydrogen production. One possible scenario is the enhanced role of X_9 as cellulose hydrolyzer and that of B_{49} as hydrogen producer. The higher hydrogen yield by $X_9 + B_{49}$ compared with $B_2 + B_{49}$ or $C_3 + B_{49}$ (Table 1) indicated that the ethanol-type fermentation metabolism does not comply with the role of cellulose hydrolyzer.

Fig. 4 – Time course of VFA in mono-cultured tests with strains X_9 (a) and time course of VFA in co-cultured tests with strains X_9 and B_{49} (b). Experiment conditions corresponding to those in Fig. 2. VFA, volatile fatty acid.

3.3. Hydrolysis of partially delignified corn stalks and yield of hydrogen by X_9

The four pretreatments, H_2SO_4 , NaOH and NH_3 soaking, and steam explosion, hydrolyzed corn stalks at a fraction of (in w/w) 15–16%, 7–13%, 13–17%, and approximately 10%, respectively (Fig. 5). The strain X_9 further hydrolyzed the pretreated corn stalks in 10–12 h to (w/w) 39–46%, 37–45%, 33–43%, and approximately 47%, respectively. Restated, the strain X_9

significantly hydrolyzed the pretreated corn stalks, a consequence attributable to the decrease in cellulose crystallinity and removal of lignin following pretreatments [18,19].

Consistent with the trend of cellulose hydrolysis, the hydrogen yield from pretreated corn stalks followed steam explosion (188 ml H_2 l⁻¹ medium) > 0.5% sulfuric acid (150 ml H_2 l⁻¹ medium) > 1.0% sodium hydroxide (80.7 ml H_2 l⁻¹ medium) > 2.0% ammonia liquor (76.2 ml H_2 l⁻¹ medium).

Additionally, the lag time for hydrogen yield from steam exploded (2 h) stalks is much less than that of sulfuric acid pretreated (>24 h). The steam explosion presents the most effective method amongst the four tested pretreatments for hydrolyzing cellulose and yielding hydrogen. Based on the data of accumulated hydrogen yield, the specific hydrogen production rate (Y_{H_2}/s) of strain X_9 is 3.4 mmol g⁻¹ steam exploded corn stalks.

4. Conclusions

This study produced directly biohydrogen from cellulosic materials using dark fermentation with selected isolates. Three isolates, B_2 , C_3 and X_9 , could yield hydrogen from microcrystalline cellulose or from CMC. The strain X_9 , identified as *C. acetobutylicum*, underwent typical butyrate-type fermentation metabolism, whereas B_2 and C_3 had so-called ethanol-type fermentation metabolism. At 37 °C and pH 5.0, the mono-culture tests with X_9 revealed much higher hydrogen yield than did other strains. Meanwhile, the co-cultures of any two of the three strains exhibited 3–4 times hydrogen yields compared with the mono-culture tests. Particularly, the co-cultures of X_9 and strain *E. harbinense* B_{49} yielded the highest hydrogen yield. The corresponding end liquid products were acetate, ethanol, and butyrate. Co-culturing these strains revealed bioaugmentation effects for promoting biohydrogen production from cellulosic materials.

The corn stalks pretreated with four processes: H_2SO_4 , NaOH and NH_3 soaking, and steam explosion were further hydrolyzed using the strain X_9 . Over 10–12 h the hydrolysis ratio reached (in w/w) 39–46%, 37–45%, 33–43%, and approximately 47%, respectively, for the four pretreated corn stalks. The yielded hydrogen followed the same order. The steam explosion + hydrolysis/fermentation with X_9 present the most effective method amongst the four tested pretreatments for hydrolyzing cellulose and yielding hydrogen.

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Fig. 5 – Cellulose hydrolysis and hydrogen yield of strain X_9 using corn stalks following four pretreatment methods at 35 °C. H – H_2 ; C – cellulose; SH – sodium hydroxide pretreatment (a); AL – ammonia liquor pretreatment (b); SA – sulfuric acid pretreatment and SE – steam explosion pretreatment (c).

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