Bioaugmented Hydrogen Production from Lignocellulosic Substrates Using Co-Cultures of *Shigella flexneri str.* G3 and *Clostridium acetobutylicum* X9

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Abstract: Bioaugmented fermentation of cellulosic substrates to produce biohydrogen *via* co-culture of isolated strains was investigated. Two mesophilic anaerobic bacterial strains, known for their ability to hydrolyze cellulosic substrates, were taken in consideration: *Shigella flexneri str.* G3, which shows high cellulolytic activity but cannot ferment oligosaccharides to bioenergy, and *Clostridium acetobutylicum* X9, able to convert microcrystalline cellulose into hydrogen. The ability of the selected strains to effectively convert different cellulosic substrates to hydrogen was tested on carboxymethyl cellulose (AVICEL), as well as pretreated lignocellulosic material such as Bermuda grass, corn stover, rice straw, and corn cob. Results showed that co-culture of *Shigella flexneri str* G3 and *Clostridium acetobutylicum* X9 efficiently improved cellulose hydrolysis and subsequent hydrogen production from carboxymethyl cellulose. Hydrogen production yield was enhanced from 0.65 mol H_2 (mol glucose)$^{-1}$ of the X9 single culture to approximately 1.5 mol H_2 (mol glucose)$^{-1}$ of the co-culture, while the cellulose degradation efficiency increased from 50% to 95%. Co-culture also efficiently improved hydrogen production from natural lignocellulosic materials (which was up to 4-5 times higher than mono-culture with X9), with the highest performance of 24.8 mmol L^{-1} obtained on Bermuda grass. The results demonstrate that co-culture of *S. flexneri* G3 and *C. acetobutylicum* X9 was capable of efficiently enhance cellulose conversion to hydrogen, thus fostering potential biofuel applications under mesophilic conditions.

Keywords: Bioaugmentation, co-culture, lignocelluloses, saccharification, biohydrogen.

1. INTRODUCTION

Production of renewable fuels such as bio-hydrogen from lignocellulosic biomass holds remarkable potential to meet the current energy demand, as well as to mitigate greenhouse gas emissions for a sustainable environment [1-2]. The use of lignocellulosic substrates is thus very promising and might provide abundant non-food feedstocks for the production of second-generation biofuels, with environmental benefits and large net energy gains [3]. In fact, biomass-derived saccharides such as glucose, cellobiose and other minor sugars, can be readily fermented by appropriate microbes into bioenergy products and other commodity chemicals [4]. Nonetheless, how to effectively convert lignocellulosic materials to sugars is the bottleneck in cellulosic biofuels industry [5-6].

Lignocellulosic biofuels can only be competitive on an industrial scale if efficient and viable technologies can be developed [7-9]. Combining hydrolysis of cellulose with simultaneous fermentation of oligo-saccharides in a single process, i.e. direct microbial conversion (DMC), is an ideal strategy for converting cellulosic biomass to bioenergy (i.e H_2 or ethanol). However, no single microorganisms/communities can implement DMC with a high efficiency [10]. Thus, a combination of high-active cellulose hydrolyzing bacteria and hydrogen-producing bacteria could result in a more efficient hydrogen production from cellulosic materials. Liu *et al*. (2003), for instance, reported that their mixed culture, comprising microbes closely affiliated with the genus *Thermoanaerobacterium*, produced 7.56 mg H_2 g^{-1} cellulose from a 5 g cellulose l^{-1} suspension, maintained at 55 ^{o}C [11]; while Liu and colleagues (2008) showed that the mixed culture of *Clostridium thermocellum* JN4 and *Thermoanaero-bacterium thermosaccharolyticum* GD17 was able to produce 1.8 mol H_2 (mol glucose)$^{-1}$ from 5 g l^{-1} carboxymethyl cellulose [12].

Although thermophilic bacteria are widely used in cellulosic-hydrogen processes (carried out at high temperatures), mesophilic organisms would help reducing operational costs, and are important for many industrial processes, typically carried out at room

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temperature [13] or slightly higher. However, cellulose hydrolysis under mesophilic conditions is generally slower and less efficient. For instance, Ren and colleagues reported that the hydrogen yield by *Clostridium populeti* from 7.8 g/l cellulose at 37 ^{o}C was 1.4 (mol glucose)$^{-1}$ [14]; *Clostridium butyricum* CGS5 exhibited H_2 production from rice husk hydrolysates with a H_2 yield of 17.24 mmol H2 (g cellulose)$^{-1}$ [15]. Lo *et al*. [16] investigated the cellulosic-hydrogen production from mixed bacterial *Clostridium* species consortia. The results showed consortia NS hydrolyzed 10 g/L CMC to produce 0.097 mmol H2/g cellulose at 35 ^{o}C.

In a previous study we reported that *Shigella flexneri str.* G3 exhibits high hydrolytic activity and oligosaccharides production capability under mesophilic conditions: oligosaccharide production yield reached 375 mg g^{-1} Avicel [13]. To our knowledge, this represents the highest oligosaccharide yield and specific rate from cellulose for mesophilic bacterial monocultures reported so far. However, no bioenergy products (i.e. hydrogen, ethanol, biodiesel) were generated by *S.* G3 [13]. On the other hand, *Clostridium acetobutylicum* X9, another strain isolated from our lab [17], produced hydrogen directly from cellulose (AVICEL) at 37 ^{o}C.

The overall aim of this study was to evaluate the effect of bioaugmentation on the ability of converting cellulose to hydrogen under mesophilic conditions. To do so, the mesophilic cellulolytic bacterium *Shigella flexneri str.* G3, which is capable of rapid and efficient production of sugars from cellulose, was put in co-culture with the hydrogen-producing *Clostridium acetobutylicum* X9, in order to investigated their joint cellulose hydrolysis and hydrogen production performance.

2. MATERIALS AND METHODS

2.1. Strains and Medium

The two bacterial strains used in this study were previously isolated at our laboratory. *Shigella flexneri str.* G3 is a gram-negative, short rod-shaped, non-motile bacterium (Figure **S1a**), which was isolated from rumen liquor [13]. It exhibits high hydrolytic activity (cellulose degradation reaching around 75%) and among the highest oligosaccharide yields from cellulose, under mesophilic conditions. Oligosaccharides generated by G3 are mainly composed of glucose (30%) and cellobiose (70%), probably related to the low β-glucosidase activity [13]. However, as already mentioned, *Shigella flexneri str.* G3 is not able to produce hydrogen. *Clostridium acetobutylicum* X9 (Figure **S1b**), a cellulosic-hydrogen producing fermentative bacteria, was isolated from a continuous flow anaerobic reactor fed with molasses (Wang *et al*., 2008). The strain has regular rod cells without flagellum and is able to rapidly utilize a large variety of pure cellulose, di-/tri-saccharides and monosaccharides to grow. A previous study showed an appreciable Avicel degradation ability [18]. Besides Avicel, X9 is also able to utilize other substrates, such as glucose, cellobiose, lactose, maltose, fructose, mannose as carbon sources to produce hydrogen. It was thus utilized for co-culture with strain G3 in fermentation tests.

The Mp medium (modified from ATCC1191 medium) was used for cultivation experiments, containing (per liter): 3.0 g of Avicel PH-101 (50 μm, HukaBiochemika 11365, Sigma-Aldrich Chemie), 1.5 g of KH_2PO_4, 4.2 g of $Na_2HPO_4{\cdot}12H_2O$, 0.5 g of NH_4Cl, 0.18 g of $MgCl_2{\cdot}6H_2O$, 1.0 g of yeast extract, 0.5 g of L-cysteine, and 1 ml of resazurin (0.2%). All the cultivation tests were performed in Vinyl Type A anaerobic chambers (Coy laboratory products, Inc., USA) containing 80% N_2, 20% CO_2 atmosphere, and operated at 37 ºC. Bacterial cultures of mid-log phase were transferred into 1ml vials containing 20% (v/v) glycerol and kept in -80°C for future use. A modified Mp medium, replacing Avicel PH-101 with 1.0 g of glucose (other gradients kept same), was used as the pre-culture medium for the two individual strains. Initial pH of medium after autoclave (121^{o}C, 15 min) was 6.8, and was not controlled nor buffered during the test.

Moreover, hydrolysis and hydrogen production were also tested on pretreated (1.2 % w/v H_2SO_4 steam-exploded) natural lignocellulosic materials, such as Bermuda grass, corn stover, rice straw, and corn cob. Pretreatment was used for lignin depolymerization, thus improving cellulose utilization. Experiments were performed using 5 g L^{-1} of such pretreated substrates, which were obtained according to the method described by Ren and colleagues [17].

2.2. Co-Culture Test

The idea of co-culture was to use strain X9 (hydrogen producer) to ferment oligosaccharides generated by G3. Prior to co-culture, the single strains *S.* G3 and *C.* X9 were cultured individually with 0.1% of glucose for 40 hours and 10 hours, respectively. Mid-log phase cells were collected, filtered, and washed

with distilled water for co-culture test. Co-culture tests were performed using the Mp medium as described in the cultivation tests. 150 mL medium were mixed with 15 ml inoculum containing 0.425 g dry cells (volume ratio of G3 and X9: 50%: 50%), and kept at 37°C for 50 h. In order to better evaluate the bioaugmented hydrogen production, mono-culture fermentation with X9 was conducted as control. In the pure culture control test the biomass was adjusted to 0.425 g dry cells.

Samples were taken every 5 hrs for a 50-hour incubation period, to determine cell biomass, pH change, hydrogen yield, cellulose degraded, saccharide yield, and liquid end products.

2.3. Analytical Procedures

Cell growth on insoluble microcrystalline cellulose was determined indirectly by measuring the total protein after cell-lysis pretreatment using a modified method described by Bradford [19]. The process was as follows: 1 ml mid-log bacterial cell culture with Avicel was collected and centrifuged at the maximum speed (14,000 x g) for 10 min at room temperature. After discarding the suspension, 300 µl mixture (0.303 g of Tris, 0.189 ml of HCl, 0.8 g of SDS dissolved in 10 ml of ultra-pure water) was added to the pellet, and incubated at 100°C for 20 min. After cooling, the supernatant was separated by centrifugation at the maximum speed (14,000 x g) at room temperature, and used to determine cellular protein content using the Bradford method [19]. A standard curve was generated using a series of concentrations of bovine serum albumin (BSA) solutions as standards. Absorbance was measured in triplicate at 595 nm after 20 min of incubation at room temperature.

Cellulose concentration was determined using the method of Huang and colleagues [20]. Residual cellulose was washed by using acetic acid - nitric acid reagent and water to remove non-cellulosic materials, as described by Updegraff [21]. Cellulose was then quantified using the phenol-sulfuric acid method [22], with glucose as the standard. Saccharides and liquid end products, such as volatile fatty acids (VFA), were determined by a Hewlett-Packard HPLC chromatograph (HP1090) equipped with a refractive index and UV detector (λ = 210nm), using a solution of 5 mN H_2SO_4 (mobile phase) as described elsewhere [23]. Avicel hydrolysis ratio % was estimated as the fraction of the cellulose that was consumed, given as a percent of the total provided as substrate. Oligosaccharide yields were calculated as the amount of oligosaccharides produced (mg) per Avicel added (g). The gas composition was measured using methods previously described by Wang *et al*. [18]. Yields of hydrogen (Y_{H2}) were indicated as moles of hydrogen produced per mole of glucose equivalents. The microcrystalline cellulose was considered as a polysaccharide with the formula $(C_6H_{10}O_5)_n$. The amount of glucose equivalents was calculated based on molecular weight of the monosaccharide molecule of microcrystalline cellulose.

2.4. Carbon Mass Balance

Carbon mass balance closure was calculated as output carbon mass divided by input carbon mass [24]:

$$Closure(\%) = \frac{carbone\ coverd\ [\sum Cout](g)}{initial\ coverd\ [\sum Cin](g)} \times 100$$

The carbon contribution from the medium components is shown in Table **S1**. The estimation of carbon mass balance for cellulose degradation on Avicel requires the information on initial and final carbon contribution, including cellulose concentrations, cell mass concentrations, soluble protein concentrations, concentrations of saccharides, cumulative gaseous carbon dioxide production (total CO_2), and byproducts (organic acids). All the parameters were measured immediately after inoculation and thereafter every five hours until the end of the cultivation. Measured methods of carbon content from all sources can be found in our previous study [13].

3. RESULTS AND DISCUSSION

3.1. Cellulose Utilization and Oligosaccharides Production

Co-culture with G3 and X9 was able to complete the fermentation process within 45 h, generating a cellulose utilization quantity of 2.85g (95%) (Figures **1** and **2**). Glucose was undetectable and cellobiose kept a trace level of 3 mg g^{-1} Avicel, suggesting that oligosaccharides were completely converted. As a comparison, the cellulose utilization ratio of X9 mono-culture reached 50% (1.5g) in 8 h and did not further increase with time. Cell proteins produced by co-culture of *S. flexneri* G3 and *C. acetobutylicum* X9 were 0.683 g l^{-1} total proteins (Figure **1**).

These results suggest that cooperation within the co-culture (G3+X9) might indeed improve substrate

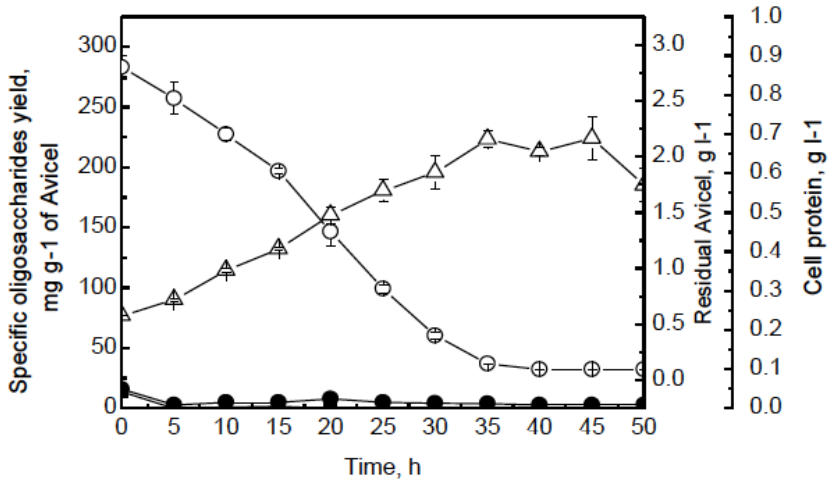

Figure 1: Kinetics of Avicel degradation over time during co-culture tests with strains G3 + X9. Specific oligosaccharides yield (■, glucose; ●, cellobiose, left Y-axis), residual cellulose (○, right Y-axis), and cellular protein (△, Y-axis) by co-cultured strains G3 + X9, grown at 3.0 g L^{-1} of Avicel PH-101, in 50 h of batch fermentation tests. Data are presented as the mean of triplicate cultures with standard deviations (error bars).

degradation efficiency, as well as the kinetics. Clearly, the incomplete utilization of the substrate (2.25g of Avicel utilization in 60 h) observed in G3 in the previous study [13] could also be related to the accumulation of inhibitory compounds in medium or cells [25-26]. In fact, various types of cellulase enzymes can be inhibited by accumulation of soluble products (glucose, cellobiose, cellotriose, etc.) *via* feedback mechanisms [27]. Thus, in the co-culture test with G3+X9, the complete conversion of oligosaccharides might have also favored hydrolytic activity, by avoiding feedback inhibition and improving continuous cellulose degradation *via S. flexneri* G3.

3.2. Hydrogen Production

As can be seen in Figure **2**, the hydrogen production of co-culture G3+X9, degrading Avicel at 37 °C and pH 6.7, reached about 25 mmol L^{-1}, corresponding to a yield of 1.50 mol H_2 (mol glucose)$^{-1}$. In comparison, hydrogen production from mono-culture X9 was significantly lower, with 6 mmol L^{-1}, corresponding to a yield of 0.65 mol H_2 (mol glucose)$^{-1}$), and diplayed a different time pattern: maximum hydrogen production was reached after 10 hours (with no evident lag-time) and then rapidly decreased, while it kept increasing until 45 h fermentation in the co-culture. So there was a clear

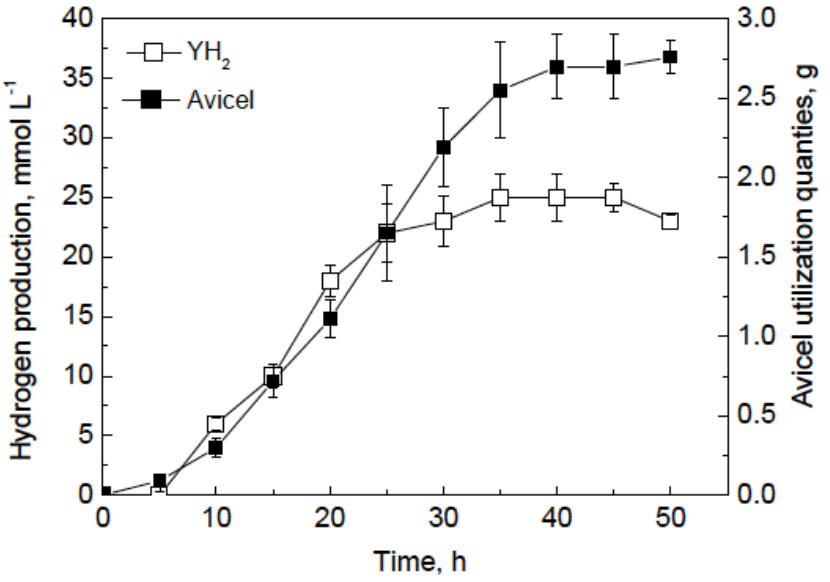

Figure 2: Hydrogen production and cellulose utilization from co-cultured strains G3 + X9 within 50 hrs of batch fermentation time, grown at 3.0 g L^{-1} of Avicel. Data are presented as the mean of triplicate cultures with standard deviations (error bars).

positive effect on the cellulose utilization / hydrogen production performances in the co-culture G3+X9. The bioaugmented hydrogen production (by X9) was seemingly enhanced by the cellulose hydrolytic role of G3, while on the other hand, the continuous conversion of oligosaccharides (to produce H2) by X9 might have favoured the hydrolytic activity of G3, thus suggesting a possible mutual benefit or cooperation of the two strains.

Previous observations showed that G3 mono-culture started cellulose degradation only after a lag phase of several hours [13]. Therefore it was probably *C. acetobutylicum* X9, which started to metabolize Avicel and to grow (producing hydrogen) in the initial stage of the co-culture. After the initial phase, however, the contribution of G3 to the co-culture seemed to become more evident, seemingly providing an excellent (additional) carbon source for X9, which was able to continue produce hydrogen until 45h (while X9 mono-culture did not show any further hydrogen production already after 10 h).

3.3. pH and Liquid End Products

The liquid end products of mono-culture *C. acetobutylicum* X9 were primarily composed of acetate and butyrate (Figure **3**), in accordance with the findings by Wang and colleagues [18]. Acetate and butyrate formation were most probably linked to the hydrogen production (by X9) *via* butyric acid metabolism. Our results indicated that more organic acids, especially butyrate, acetate and propionate were produced from cellulose degradation and oligosaccharides fermentation during co-culture tests with *G3*+X9 (probably due to the a more efficient substrate conversion). Acetate and butyrate showed similar concentration, which increased approximately 3.5 times (each), from less than 350 mg L^{-1} with X9 to about 1200 mg L^{-1} with the co-culture G3+X9, respsctively. Propionate, a typical metabolite from cellulose degradation, was only detected in the co-culture fermentation. Lactate, which is an inhibitor of cellulose degradation [28-29], was also detected during co-culture fermentation, but at a lower concentration compared to the other metabolites. The presence of lactate and propionate in the liquid end products was also observed in mono-culture with G3 [13].

The suspension pH dropped from initial 6.7 to approximately 5.0 in the mono-culture of X9. This pH drop corresponded to the starting phase for hydrogen production. Similarly, during co-culture tests, the pH rapidly decreased to pH 4.5 within 20 h and did not change significantly in the remaining 30 h fermentation, thus finally reaching pH 4.3.

3.4. Carbon Mass Balance

Carbon balance (C-balance) of co-cutlure G3+X9 was assessed by taking into account Avicel consumption, production of oligosaccharides, liquid end products and CO_2, as well as cell biomass (as total protein). As can be observed in Table **1**, carbon closure decrease from initial 99.0±0.01% (observed at 5 h) to 92.8±0.35% (after 50 h). This trend was in good agreement with the previous study by Wang and colleagues [13], based on strain G3. However,

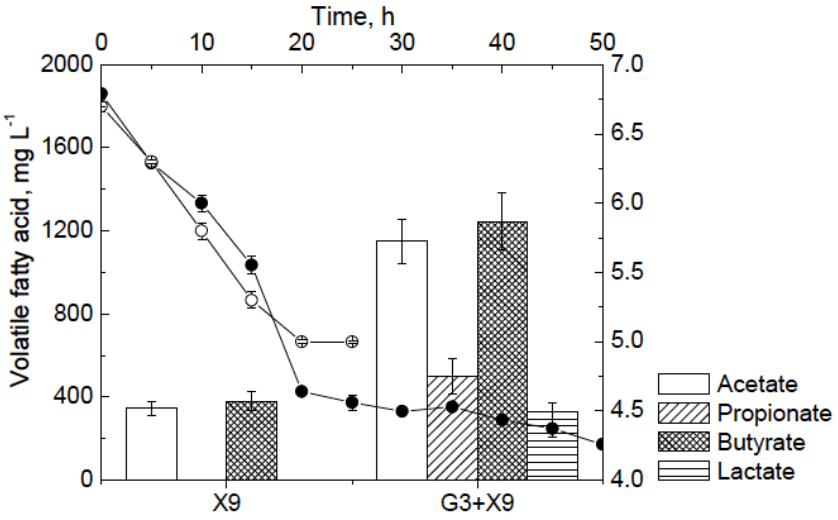

Figure 3: Volatile fatty acid (VFA) and pH profile from mono-culture X9 (o) and co-cultured strains G3 + X9 (●), grown at 3.0 g L^{-1} of Avicel, within 50 h of batch fermentation. Data are presented as the mean of triplicate cultures with standard deviations (error bars).

Table 1: Average carbon mass allocation for cellulosic-hydrogen runs on Avicel PH-101 substrate by co-culture of *S.* G3 & *C.* X9. Results are shown with ±1 standard deviation values for triplicate tubes after 50 h of incubation and are expressed as g C per Liter Mp medium

Run time	Avicel	Media	Cell mass	Soluble protein	Saccharides	CO2	VFA	C-closure,%
0	1.29±0.01	0.16±0.04	0.23±0.005	0	0	0	0	100.0
5h	1.22±0.02	0.13±0.05	0.23±0.005	0.01±0.007	0	0.01±0.001	0.08±0.002	99.0±0.01
10h	1.00±0.01	0.08±0.01	0.25±0.01	0.01±0.005	0.01±0.001	0.05±0.001	0.35±0.03	95.9±0.12
15h	0.80±0.05	0.04±0.003	0.26±0.01	0.02 ±0.001	0.03±0.005	0.08±0.003	0.37±0.01	95.4±0.15
20h	0.67±0.02	0.02±0.005	0.28±0.05	0.02±0.003	0.03±0.002	0.14±0.005	0.44±0.03	95.2±0.12
25h	0.45±0.03	0	0.29±0.02	0.03±0.001	0.02±0.008	0.15±0.02	0.60±0.05	93.0±0.20
30h	0.27±0.02	0	0.31±0.02	0.03±0.002	0.02±0.005	0.15±0.08	0.76±0.04	95.7±0.20
35h	0.11±0.08	0	0.33±0.03	0.05±0.002	0.02±0.001	0.13±0.02	0.95±0.02	94.6±0.45
40h	0.07±0.005	0	0.33±0.02	0.06±0.002	0.01±0.003	0.10±0.003	1.06±0.01	94.0±0.38
45h	0.06±0.005	0	0.30±0.05	0.08±0.004	0.01±0.003	0.10±0.005	1.07±0.01	94.2±0.34
50h	0.06±0.001	0	0.26±0.04	0.13±0.03	0.01±0.001	0.10±0.002	1.06±0.02	92.8±0.35

differently from the previous study on G3, the carbon balance analysis of the co-culture X9+G3 indicated that VFAs (and not the oligosaccharides, as in G3) were the dominant component during the fermentation. About 82.2% of the initial substrate was converted to liquid end products, such as acetate, butyrate, propionate and lactate, and the rest was converted into biomass, trace sugars, proteins and CO_2. As a comparison, during the fermentation with X9 mono-culture, around 28.8% of initial substrate was converted into VFAs (with a carbon closure of 88.6±0.43% at the end of fermentation). The effective conversion of the substrate into VFAs such as acetate and butyrate (by far the biggest fraction of the liquid end products) by the co-culture G3+X9, without accumulation of oligosacharides, might explain the enhanced overall cellulose conversion efficiency to hydrogen.

3.5. Hydrolysis of Pretreated Natural Lignocellulosic Substrates and Hydrogen Production

Mono-culture of *C. acetobutylicum* X9 and the co-culture of *S. flexneri* G3 and *C. acetobutylicum* X9 were also grown on pretreated lignocellulosic materials. Hydrogen production obtained from the different pretreated lignocellulosic substrates by co-culture G3 + X9 (Figure **4**) was: 24.8 mmol L^{-1} medium (Bermuda grass) > 19.2 mmol L^{-1} medium (rice straw) > 17.8 mmol L^{-1} medium (corn stover) > 14.4 mmol L^{-1} medium (corn cob). Noticeably, the hydrogen production from Bermuda grass was similar to that from Avicel (25.3 mmol L^{-1}; Figure **2**). Most likely, the reason for the highest hydrogen production from Bermuda grass was that it has the lowest lignin component (~2%) among the four test substrates [30], thus being relatively easier to be decomposed.

The hydrogen yield from natural lignocellulosic material by co-culture G3 + X9 was on average 4-5 times higher than mono-culture with X9. Moreover, hydrogen was not detected from pretreated corn stover when strain X9 was cultured alone, while it was produced in co-culture conditions, thus confirming that the co-culture could enhance the hydrogen production ability from lignocellulosic biomass. This preliminary results suggested that G3 and X9 may provide useful combinations of metabolic pathways for the processing of complex waste material (and the degradation of impurities and/or inhibitors), thereby supporting a more efficient decomposition of substrate. Similar conclusions were also reported by other authors [31-34].

4. CONCLUSIONS

This study showed the possibility to effectively enhance biohydrogen production from different cellulosic materials *via* co-culture of *S. flexneri* G3 and *C. acetobutylicum* X9. Maximum hydrogen yield on Avicel reached 1.5 mol H_2 (mol glucose)$^{-1}$, with a cellulose hydrolysis ratio as high as 95% in 45 h. The corresponding end liquid products were butyrate and acetate, followed by propionate and lactate. Moreover, co-culturing also revealed bioaugmentation effects on biohydrogen production from other cellulosic

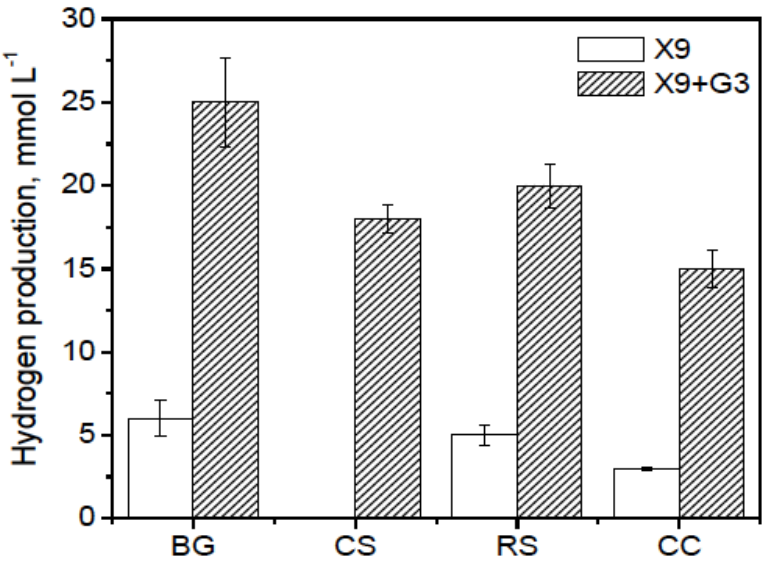

Figure 4: Hydrogen production from pretreated natural lignocellulosic material, using mono-culture X9 and co-cultured strains (G3 + X9) grown at a substrate concentration of 5.0 g L^{-1}. BG = Bermuda gass; CS = corn stover; RS = rice straw; CC = corn cob.

substrates, such as pretreated Bermuda grass, corn stover, rice straw and corn cob, with a hydrogen production ranging from 14.4 mmol L^{-1} to 24.8 mmol L^{-1}. Co-culture efficiently improved hydrogen production by 4-5 times compared to mono-culture with X9. These results indicated that bioaugmented cultures show an enhanced ability in the conversion of several pretreated lignocellulosic substrates, with a hydrogen production that can be comparable to the one obtained from (3 g L^{-1}) microcrystalline cellulose. In conclusion we can affirm that co-culture of *S. flexneri and* G3 & *C. acetobutylicum* X9 was capable to efficiently enhance cellulose conversion to hydrogen, thus fostering potential biofuel applications under mesophilic conditions.

ACKNOWLEDGEMENTS

This research was supported by National Natural Science Foundation of China (NSFC, Grant No. 51111140388, No. 51208496), by Science Fund for Creative Research Groups of the National Natural Science Foundation of China (Grant No. 51121062) by China Postdoctoral Science Foundation (No. 2012M510574 and No. 2013T60182). This work was also supported by the State Key Laboratory of Urban Water Resource and Environment (grant no. 2010DX11 and no. 2011TS09).

SUPPLEMENTAL MATERIALS

The supplemental materials can be downloaded from the journal website along with the article.

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Received on 14-04-2014 Accepted on 17-05-2014 Published on 30-05-2014

DOI: http://dx.doi.org/10.6000/1929-6002.2014.03.02.1