Dark H₂ fermentation from sucrose and xylose using H₂-producing indigenous bacteria: Feasibility and kinetic studies
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Introduction
Biomass energy has become one of the major global energy alternatives to fossil fuels. Converting inexpensive waste biomass feedstock (e.g., agricultural wastes) into bioenergy (e.g., ethanol, biodiesel, and hydrogen) is a common trend. Biomass feedstock contains a large amount of cellulosic components (e.g., cellulose, hemicellulose, and lignin), which are not readily assimilable to most energy-producing bacteria (for instance, yeast or H₂-producing acidogenic bacteria). Therefore, a two-stage conversion is more feasible, in which the cellulosic materials are first hydrolyzed via physico-chemical or biological means, followed by a fermentative energy conversion step. Hydrogen is considered as an ideal energy carrier of the future since it is clean, recyclable and efficient. Biological production of hydrogen is the most environmental friendly and sustainable hydrogen-producing method. The most effective fermentative H₂ producers belong to anaerobic acid-forming bacteria (such as Clostridium sp.). Meanwhile, facultative anaerobes (such as Enterobacter sp.) are also known to be capable of producing H₂ via fermentation of organic substrates. Those microorganisms possess dual functions of generating H₂ and decontaminating organic pollutants in the environment. We have isolated several highly efficient bioH₂-producing bacterial strains from mixed cultures, such as Cl. Butyricum and Cl. Pasteurianum, which are known effective H₂ producers from organic substrates (esp. carbohydrates). In this study, the efficiency of fermentative conversion of sucrose (representing hexose) and xylose into H₂ was examined with seven H₂-producing pure strains isolated from high-rate H₂-producing system. This work aimed to assess the feasibility of using those pure strains in producing H₂ from hydrolyzed cellulosic materials (containing hexose and pentose) as the feedstock.

Results and discussion
Predominant bacterial species in two continuous dark H₂ fermentation cultures using different sugar
substrates were analyzed with PCR-DGGE method based on the separation of amplified same length fragments of genes coding for 16S rRNA. Figure 1a shows the sludge microbial populations collected form a sucrose-feeding continuous stirred H₂ producing reactor operated under a short HRT of 0.5 h and a sucrose concentration of 20-40 g COD/l. In addition to two unidentified bands (Lane C, Fig. 1a), bacterial community structure of the sludge was mainly composed of three bacterial species, namely *Cl. Pasteurianum*, *Cl. Butyricium*, and *Klebsiella pneumoniae*. In particular, *Cl. Pasteurianum* could play a major role in sucrose fermentation H₂ production and its cell concentration was high. Other bacterial species such as *Klebsiella oxytoca*, *Streptococcus* sp., *Escherichia* sp., *Pseudomonas* sp., *Dialister* sp., and *Bacillus* sp. were also found occasionally in the reactor. On the other hand, bacterial community analysis on the xylose-feeding reactor indicates a significantly different microorganism structure as shown in Fig. 1b. A distinct band representing *Cl. butyricum* was present in the sludge samples collected from cultures operated at different HRT (0.5-12 h) and appeared to be the dominant H₂-producing species in xylose-feeding cultures. Another clostridial species, *Cl. Celerecrescens*, was also identified in the sludge, but had a lower intensity and nearly disappeared when the culture was operated at HRT = 1 h. It seems that *Cl. Butyrium* was a more dominant population in the H₂-producing culture. The bacterial composition seemed to change with HRT, as *Bifidobacterium* sp. and *Olsenella* sp. were found when the reactor was operated at a high dilution rate (short HRT), while *K. oxytoca* and *Pseudomonas* sp. existed in the culture operated at a long HRT. Most of the aforementioned non-clostridia bacteria were not hydrogen producers, but might contribute to degradation of the carbon substrate.

Fig.1 16S rDNA fragments DGGE profiles of sludge bacterial composition from (a) a sucrose-feeding continuously stirred hydrogen producing reactor operated at 0.5 h HRT, 35 ℃, and sucrose concentration of 20-40 g COD/l. (b) a xylose-feeding continuously stirred hydrogen producing reactor operated at 35 ℃, xylose concentration of 20 g COD/l and a HRT of (from left to right) 12, 8, 4, 2, 1, and 0.5 h.

The isolates *Cl. Butyricum* CGS2, *Cl. Butyricum* CGS5, and *Klebsiella* sp. were able to produce H₂ from
xylose (Fig. 2a). In particular, *Cl. Butyricum* CGS5 displayed the best H₂ production activity. In contrast, the four *Cl. Pasteurianum* strains could not utilize xylose for growth. Figure 2a also shows that the shake-flask cultures gave better cell growth and H₂ production performance than the static cultures. This indicates the importance and necessity of employing mechanical shaking to enhance mass transfer efficiency. *Cl. Butyricum* CGS5 had the highest H₂ production rate and yield of 212.5 ml/h/l and 0.73 mol H₂/mol xylose, respectively, when the xylose concentration was 20 g COD/l (Fig. 2b).

For H₂ production from sucrose, all the seven pure isolates were able to utilize sucrose for cell growth and H₂ production (Fig. 3a), suggesting that sucrose is a more easily assimilable substrate than xylose for the H₂-producing bacterial isolates. The *Cl. Pasteurianum* strains exhibited much better H₂ production efficiency than the *Cl. butyricum* strains (Fig. 3a). *Cl. Pasteurianum* CH4 exhibited the best H₂-producing performance with a H₂ production rate and yield of 512 ml/h/l and 3.15 mol H₂/mol sucrose (or 1.58 mol H₂/mol hexose), respectively (Fig. 3a). *Cl. Pasteurianum* CH4 gave the highest H₂ production rate (569 ml/h/l) and yield (4.14 mol H₂/mol sucrose or 2.07 mol H₂/mol hexose) when sucrose concentration was 40 g COD/l (Fig. 3b).

![Graphs showing H₂ production from xylose and sucrose](image)

Fig. 2  (a) H₂ production from pure isolates using xylose as the sole carbon substrate under shake flask or static incubation (b) Effect of xylose concentration on H₂ production performance of *Cl. Butyricum* CGS5
Fig. 3 (a) H₂ production from pure isolates using sucrose as the sole carbon substrate under shake flask or static incubation (b) Effect of sucrose concentration on H₂ production performance of *Cl. Pasteurianum* CH₄

The kinetics describing cell growth and H₂ production is valuable information to bioreactor design and process scale-up for bioH₂ operations. Unfortunately, very little work has been devoted to identifying such kinetic characteristics, especially for pure H₂-producing bacterial strains. The dependence of cell growth rate of strain CGS5 on xylose concentration was described by Monod-type (Eqn. 1) kinetic models (\( \mu = \frac{\mu_{\text{max}} S}{K_S + S} \) (Eqn. 1)). The Monod-type model fitted the experimental results with a R² value of 0.881 (Fig. 4a). The estimated parameters for Monod-type model (i.e., \( \mu_{\text{max}} \) and \( K_S \)) were 0.15 h⁻¹ and 0.67 g COD/l, respectively. In addition, the dependence of H₂ production rate on xylose concentration was also described by Michaelis-Menten (M-M) model (\( v = \frac{v_{\text{max}} S}{K_m + S} \)) (Eqn. 2) The model could describe the experimental data quite well with a R² value of 0.952 (Fig. 4b). The estimated values of \( v_{\text{max}} \) and \( K_m \) from M-M model were 0.15 l/h/g VSS and 0.5 g COD/l, respectively. To our best knowledge, this is the first presentation revealing the growth and H₂-producing kinetics of a *Cl. butyricum* species on xylose. The dependence of cell growth and H₂ production on sucrose concentration is depicted in Fig. 5a and 5b, respectively. The \( \mu_{\text{max}} \) and \( K_S \) values were 0.31 h⁻¹ and 4.39 g COD/l, respectively. The H₂ production kinetic parameters (i.e., \( v_{\text{max}} \) and \( K_m \)) were 0.29 l/h/g VSS and 3.7 g COD/l, respectively.

Moreover, a H₂ yield coefficient (\( \alpha \)) representing the correlation between H₂ production and biomass gain was calculated based on kinetic model for growth-associated products

\[
\frac{1}{X} \frac{dC_{H_2}}{dt} = \alpha \mu = \alpha \left( \frac{1}{X} \frac{dX}{dt} \right) \quad \text{or} \quad \frac{dC_m}{dX} = \alpha \quad \text{(Eqn. 4)},
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where \( C_{H_2} \) represents H₂ concentration (mol), \( X \) represents cell concentration (g VSS/l), \( \mu \) represents specific growth rate (h⁻¹). The model fits the data quite well (with a R² value of over 0.910) and the calculated \( \alpha \) value was 0.041 and 0.039 mol/g VSS for xylose and sucrose, respectively. This shows that although using sucrose as the substrate gave higher cell growth and H₂ production rates, the biomass-based yield for H₂ production was very similar,
indicating that the two Clostridium strains could produce H₂ from the two carbon substrates with similar conversion efficiency, even though the type of carbon source could significantly affect the kinetic characteristics of H₂ production (e.g., the $v_{max}$ and $K_m$ values).

**Fig. 4** Effect of xylose concentration on (a) Specific cell growth rate and (b) Specific H₂ production rate of Cl. butyricum CGS5.

**Fig. 5** Effect of sucrose concentration on (a) Specific cell growth rate and (b) Specific H₂ production rate of Cl. Pasteurianum CH4.