Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–9, 2017

www.elsevier.com/locate/jbiosc

Evaluating new bio-hydrogen producers: *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY

Yee Meng Wong,[1] Ta Yeong Wu,[2] Tau Chuan Ling,[3] Pau Loke Show,[4] Sze Ying Lee,[4] Jo-Shu Chang,[5] Shaliza Ibrahim,[6] and Joon Ching Juan[1,7,*]

Laboratory of Advance Catalysis and Environmental Technology, School of Science, Monash University, Jalan Lagoon Selatan, 46150 Bandar Sunway, Malaysia,[1] Chemical and Sustainable Process Engineering Research Group, School of Engineering, Monash University, Jalan Lagoon Selatan, 46150 Bandar Sunway, Malaysia,[2] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia,[3] Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Malaysia,[4] Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan 701, Taiwan,[5] Department of Civil Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia,[6] and Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia[7]

Received 15 September 2017; accepted 15 December 2017
Available online xxx

Three newly discovered H_2 producing bacteria namely *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY originated from landfill leachate sludge have demonstrated highly efficient H_2 production. The maximum H_2 production attained from these isolates are in the descending order of strain *C. perfringens* strain JJC > *C. bifermentans* strain WYM > *Clostridium* sp. strain Ade.TY with yield of 4.68 ± 0.12, 3.29 ± 0.11, and 2.87 ± 0.10 mol H_2/mol glucose, respectively. The result has broken the conventional theoretical yield of 4 mol H_2/mol glucose. These isolates were thermodynamically favourable with Gibbs free energy between −33 and −35 kJ/mol (under process conditions: pH 6, 37 °C and 5 g/L glucose). All three isolates favour butyrate pathway for H_2 production with the ratio of acetate and butyrate of 0.77, 0.65 and 0.80 for strain JJC, WYM and Ade.TY, respectively. This study reported provides a new insight on the potential of unique bacteria in H_2 production.

[**Key words:** Thermodynamics; Biohydrogen; *Clostridium* spp.; Hydrogenase; Dark fermentation]

Hydrogen (H_2) is a clean and environmental friendly fuel carrier because combustion of H_2 produces water as the only end product (1). Biological H_2 production is a sustainable process because it can be produced from inexpensive waste biomass such as agricultural and organic-rich industrial waste via dark fermentation (2–4). Dark fermentation is an anaerobic process that converts biomass into biological H_2 in the absence of light (5,6). It is a light-independent process and hence the configuration of the bioreactor is simpler and cheaper (7). Besides, it can utilize a wide range of carbohydrates as substrates to produce bio-H_2. Therefore, dark fermentation can be easily integrated into waste management to achieve waste reduction and bio-energy production concurrently (2–4).

In dark fermentation, several H_2 producing bacteria (HPB) such as *Clostridium* sp., *Bacillus* sp., *Klebsiella* sp. and *Enterobacter* sp. and *Ethanoligenens* sp. have been isolated from natural environment (8–18). Among the reported the HPB, *Clostridium* spp. are the most popular H_2 producers due to its high efficiency in H_2 production (9,10,12,14,15,17,18). They are obligate anaerobes that produce H_2 as well as volatile fatty acids and alcohols such acetate, butyrate, lactate, formate, ethanol and butanol that have industrial applications. Up to date, the highest H_2 yield was reported at 3.35 mol H_2/mol glucose from *Clostridium* sp. DMHC-10 (13). In theory, a maximum of 12 mol of H_2 can be produced from each mole of glucose, as described in Eq. 1. This would mean that the highest reported H_2 yield is only about 28 % and thus there is desirable to uncover more efficient H_2 producer.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (1)$$

Landfill leachate, as the name implies, is the garbage juice produced during the decomposition of organic waste within the landfill. Since active landfill sludge contained diverse microflora, we have found inoculum from landfill sludge was highly efficient for H_2 production (19). Three different types of new H_2-producing bacteria has been isolated and identified namely *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY (20–22). Based on the gene annotation, these bacteria possess hydrogenase which is the key feature for H_2 production. *Clostridium* sp. strain Ade.TY contains a unique hydrogenase (energy-converting hydrogenase), which is commonly found in archaea but it is uncommon in *Clostridium* sp. (23). Other than that, this strain also contains additional dimeric-periplasmic (Fe) hydrogenase and two (Ni–Fe) hydrogenases. For strain JJC, it contains two different types of hydrogenases which are (Fe) hydrogenase *HydA* and a dimeric cytoplasmic (Fe) hydrogenase. Meanwhile, strain WYM contains a dimeric (Ni–Fe) hydrogenase. In this study, we have investigated these H_2

* Corresponding author at: Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel.: +60 17 325 8288; fax: +60 3 7967 6960.
E-mail address: jcjuan@um.edu.my (J.C. Juan).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.12.012

producer isolates from landfill leachate under dark fermentation, as depicted in Fig. 1.

MATERIALS AND METHODS

Isolation of bacteria strains and growth conditions Sludge of landfill leachate was collected from Jeram Sanitary Landfill in Selangor, Malaysia. The sludge was pretreated at 65 °C for duration of 30 min. The H_2-producing bacteria was isolated using enrichment method as described by Tolvanen et al. (24) with slight modifications. Eight 30 mL serum bottles were arranged in series and each serum bottle was filled with 15 mL of the reaction medium. Then, they are connected using tubing (Cole–Parmer tygon tubing) with needles (0.8 × 38 mm and 0.6 × 25 mm) fixed at either ends. To initiate the enrichment process, 0.15 % w/v of sludge was inoculated to the first bottle. The enriched medium was serial diluted and inoculated onto Columbia horse blood agar (CM0331, Oxoid) and reinforcement clostridia agar (CM0151, Oxoid). The agars were prepared as the modified Hungate roll tube in 30 mL serum bottles (25). The inoculated agar was incubated anaerobically for 48 h. The colonies formed were re-streaked at least three times to obtain pure cultures.

Morphological test The cell morphology was performed using the standard gram staining and spore staining method (Merck). The morphological morphology was observed with a light microscope (Olympus BX51).

Genome project accession numbers The H_2-producing isolates were identified using whole genome sequencing with Illumina Mi-seq. The draft genome sequences are deposited in the NCBI genome project under the accession number as follows: *C. perfringens* strain JJC (AWRZ00000000.1), *C. bifermentans* strain WYM (AVSU00000000.1) and *Clostridium* sp. strain Ade.TY (AVSV00000000.1) (20–22).

H_2 production in batch fermentation Triplicate batch fermentations were performed in 200 mL serum bottles with 150 mL of media. To each serum bottle, 2 % v/v inoculum corresponding to 10^9 c.f.u was added in to the fermentation medium according to our previous work (19). Fermentations were tested in the effects of: (i) initial pH (pH 4, 5, 6, 7 and 8) and (ii) temperatures (25, 30, 37, 45 and 50 °C).

Analysis The concentration of glucose and metabolites were analysed with Agilent HPLC system (1200 series) equipped with refraction index detection and the column Animex Hi-Pex H (300 × 7.7 mm, Agilent). The temperature of column and detector were set at 65 and 55 °C, respectively. The biogas produced was collected using water displacement method. The yield and composition was analysed with gas chromatography equipped with thermal conductivity detector (TCD) and 2.7 m Hayesep Q column. The carrier gas, helium, was flow at the rate of 2 mL/min. Temperatures of injector, detector and oven were set at 100, 150 and 60 °C, respectively.

Kinetics and thermodynamic analysis Cumulative volume of H_2 was fitted with the modified Gompertz equation defined as Eq. 2 (26–28):

$$H = H_{\max}\left\{ -e\left[\frac{R_{\max}.e}{H_{\max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

where H is the cumulative H_2 production (mol H_2/mol glucose), $H_{\max}$ is the maximum H_2 production (mol H_2/mol glucose), $R_{\max}$ is the maximum H_2 production rate (mol H_2/mol glucose/h), λ is the lag phase time (h) and t is the incubation time (h), and $R_{\max}.e/H_{\max}$ represents the rate constant (k). The modified Gompertz equation was used to fit the cumulative H_2 data, using the OriginPro 8.5.

According to Fabiano and Perego (16), activation enthalpy of fermentation (ΔH) and thermal deactivation can be represented by the modified Arrhenius equation as defined in Eqs. 3 and 4:

$$\ln H_{\max} = \ln\ (A.X.Y) - \frac{\Delta H}{RT}, \quad T < T_{\text{opt}} \quad (3)$$

$$\ln H_{\max} = \ln\ (B.X.Y) - \frac{\Delta H^*}{RT}, \quad T > T_{\text{opt}} \quad (4)$$

where $H_{\max}$ is the maximum H_2 productivity from Eq. 3, A and B are the Arrhenius pre-exponential factors, X is the cell mass concentration (g/L), Y is the H_2 yield per unit cell mass (mol H_2/g cell mass), R is the ideal gas constant (8.3144621 J/K/mol) and T is temperature in Kelvin (K). The thermal deactivation enthalpy (ΔH_d) is determined using in Eq. 5.

$$\Delta H_d = \Delta H + \left|\Delta H^*\right| \quad (5)$$

Activation entropy of fermentation (ΔS) and thermal deactivation (ΔS_d) were determined using the following Eqs. 6 and 7 that derived from Eyring and Arrhenius equations (16).

$$\Delta S = R\left(\ln\frac{Ah}{k_b T}\right) \quad (6)$$

$$\Delta S_d = R\left(\ln\frac{Bh}{k_b T}\right) \quad (7)$$

where A and B are the Arrhenius pre-exponential factors, h is the Planck's constant (6.63×10^{-34} J s) and k_b is the Boltzmann's constant (1.38×10^{-23} J/K).

The Gibbs free energy, ΔG, was calculated using Eq. 8.

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

where ΔH is the activation enthalpy obtained from Eq. 5 whereas ΔS is the activation entropy obtained from Eq. 7.

RESULTS AND DISCUSSION

Characterisation of H_2 producing isolates The three isolates were identified as gram-positive and rod-shaped bacteria. Endospore staining revealed that strain JJC and WYM are endospore-forming bacteria but not strain Ade.TY, as shown in Fig. 2. Based on whole genome sequencing, heat plot from multiple genome alignment revealed that strain JJC is a *C. perfringens* and strain WYM is a *C. bifermentans*. However, multiple genome alignment shows that strain Ade.TY is a *Clostridium* species but it does not align with any existing genome sequences and hence suggests that strain Ade.TY could be a new species. The relationship of the isolates with their closely related species is represented in a phylogenetic tree based on 16S rRNA sequences displayed in Fig. 3. The phylogeny confirmed that strain JJC and WYM are *C. perfringens* and *C. bifermentans*, respectively. As for strain Ade.TY, it brunched away from the closely related species and hence further indicates that it may be a new H_2-producing species.

Effect of initial pH on H_2 production Hydrogen production from the isolates varies with initial pH. The results demonstrated

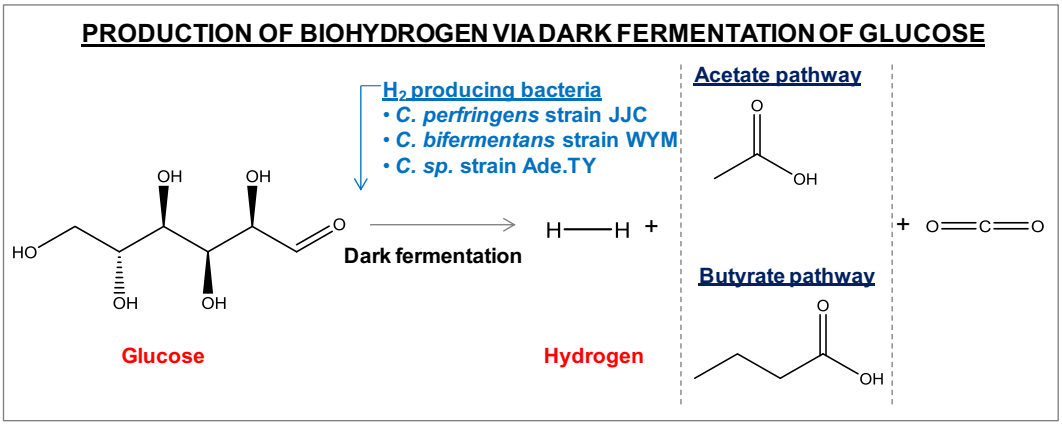

FIG. 1. Biohydrogen production scheme proposed in this work.