

# Critical analysis of hydrogen production from mixed culture fermentation under thermophilic condition (60 °C)

Hang Zheng<sup>1,2</sup> · Raymond J. Zeng<sup>3</sup> · Cathryn O'Sullivan<sup>1,4</sup> · William P. Clarke<sup>1</sup>

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**Abstract** Bio-hydrogen production from mixed culture fermentation (MCF) of glucose was studied by conducting a comprehensive product measurement and detailed mass balance analysis of their contributions to the final H<sub>2</sub> yield. The culture used in this study was enriched on glucose at 60 °C through a sequential batch operation consisting of daily glucose feeds, headspace purging and medium replacement every third day in serum bottles for over 2 years. 2-Bromoethanesulfonate (BES) was only required during the first three 3-day cycles to permanently eliminate methanogenic activity. Daily glucose feeds were fully consumed within 24 h, with a persistent H<sub>2</sub> yield of 2.7 ± 0.1 mol H<sub>2</sub>/mol glucose, even when H<sub>2</sub> was allowed to accumulate over the 3-day cycle. The measured H<sub>2</sub> production exceeded by 14 % the theoretical production of H<sub>2</sub> associated with the fermentation products, dominated by acetate and butyrate. Follow-up experiments using acetate with a <sup>13</sup>C-labelled methyl group

showed that the excess H<sub>2</sub> production was not due to acetate oxidation. Chemical formula analysis of the biomass showed a more reduced form of C<sub>5</sub>H<sub>11.8</sub>O<sub>2.1</sub>N<sub>1.1</sub> suggesting that the biomass formation may even consume produced H<sub>2</sub> from fermentation.

**Keywords** Bio-hydrogen · Excess H<sub>2</sub> production · Stable isotope · Biomass formation · Mixed culture fermentation

## Introduction

The demand for alternative energy sources is growing as fossil resources become scarcer and the effects of climate change become apparent. H<sub>2</sub> is regarded as a sustainable transportation fuel because of its high energy density (142 kJ/g, which is 2.75-fold higher compared to that of fossil fuel; Kim and Kim 2011) and its pollution-free end product (H<sub>2</sub>O) after combustion and it is the ideal fuel for highly efficient electrochemical fuel cells, which is starting to be widely applied in motor vehicles. Markets reflect these advantages with the value of H<sub>2</sub> being more than 10 times that of methane, on a chemical oxygen demand (COD) basis (Clarke and Alibardi 2010). However, H<sub>2</sub> is often thought as a non-renewable energy carrier because it is primarily made from hydrocarbon gases or coal in current industrial-scale plants. There are numerous avenues for producing H<sub>2</sub> renewably, including photo-driven electrolysis where light is captured either by photovoltaic cells or biologically by anaerobic algae or photo-fermenting heterotrophic bacteria. H<sub>2</sub> can also be produced by dark fermentation.

All of the biological H<sub>2</sub> production processes occur at moderate temperature and pressure which means they can be disseminated and co-located with organic sources. Of all these processes, dark fermentation is the simplest and widely used

✉ Raymond J. Zeng  
rzeng@ustc.edu.cn

✉ William P. Clarke  
william.clarke@uq.edu.au

<sup>1</sup> Centre for Solid Waste Bioprocessing, School of Civil Engineering, The University of Queensland, Brisbane 4072, Queensland, Australia

<sup>2</sup> Present address: School of Earth Sciences, The University of Queensland, Brisbane 4072, Queensland, Australia

<sup>3</sup> CAS Key Laboratory of Urban Pollutant Conversion, Department of Chemistry, University of Science & Technology of China, Hefei 230026, China

<sup>4</sup> Present address: CSIRO Agriculture, Underwood Ave, Floreat, WA, Australia

process. Dark fermentation vessels are designed for maximum volume rather than maximum surface area, as required for photo-driven metabolism. The major disadvantage of dark fermentation is the low conversion efficiency from organic substrate to H<sub>2</sub>. Organic solid waste is primarily composed of food waste, garden waste, and paper and can be reasonably approximated as carbohydrate, which is studied by using glucose as model substrate. The theoretical maximum H<sub>2</sub> yield from glucose fermentation is 4 mol H<sub>2</sub>/mol glucose, from the complete conversion of glucose to acetate (Nandi and Sengupta 1998):



However, only a few studies have reached this theoretical maximum yield, through the use of pure cultures (Bastidas-Oyanedel et al. 2012; Dietrich et al. 1988; Schroder et al. 1994; Soutschek et al. 1984). Reported hydrogen yields from mixed culture systems are in the range of 1.6–2.4 mol H<sub>2</sub>/mol hexose (Hawkes et al. 2002; Li et al. 2008; Zhang et al. 2015; Zhao et al. 2008) due to the production of more reduced products, e.g. ethanol, lactate and butyrate (Li and Fang 2007). Although the pure culture systems can give a higher H<sub>2</sub> yield, mixed culture systems improve the stability of the process and can be maintained in cheaper, non-sterile conditions (Bastidas-Oyanedel et al. 2015; Hallenbeck and Ghosh 2009; Kleerebezem et al. 2015; Kleerebezem and van Loosdrecht 2007; Tapia-Venegas et al. 2015). While the mixed culture systems can be operated without sterilization, H<sub>2</sub> yields of those systems are almost impossible to predict as the metabolic pathways of a mixed culture system are normally a combination of various fermentation pathways.

Based on thermodynamic calculations, H<sub>2</sub> production becomes thermodynamically more favourable when operating conditions such as temperature are optimized, which means higher H<sub>2</sub> yield could be obtained (Zhang et al. 2014; Zheng et al. 2015). Thereby strategies such as increasing operating temperature and reducing H<sub>2</sub> partial pressure have been tested in lab-scale systems to enhance H<sub>2</sub> yields from fermentation processes (Ciranna et al. 2014; de Kok et al. 2013; Kraemer and Bagley 2007; Mandal et al. 2006; Mizuno et al. 2000; Nath and Das 2004; Yokoyama et al. 2007). To better understand the effects of process parameters, common fermentation products, such as volatile fatty acids (VFAs) and alcohols, were measured for studying the metabolic pathways. However, only a few of them (Kotsopoulos et al. 2006) have conducted detailed mass balance evaluation to understand the actual H<sub>2</sub> yield contributions from those pathways.

Besides mass balance evaluation of common metabolic pathways, other non-common metabolic pathways and their effects on final H<sub>2</sub> yield were seldom considered, e.g. biomass, polyhydroxyalkanoates (PHA) production and analytical errors under higher operating temperature. For example,

the forms of biomass also determine if the biomass growth process is a H<sub>2</sub>-yielding or H<sub>2</sub>-consuming process and vapour condensation in an airtight gas sampling syringe will cause overestimation of gas productions (Kim and Daniels 1991).

This study focused on the comprehensive measurement of H<sub>2</sub> fermentation products at 60 °C MCF and depth analysis of their contributions to the final H<sub>2</sub> yield. Also, a kinetic study and an isotope-labelled substrate feeding study were conducted to uncover the possible high H<sub>2</sub>-yielding pathways. The outcome of this study is expected to provide a comprehensive evaluation methodology for better understanding of MCF under an elevated temperature condition.

## Materials and methods

### Inoculum and substrates

H<sub>2</sub>-producing organisms were enriched from the leachate of an anaerobic digester treating mixed organic waste at a temperature of 47 °C. The culture was enriched through successive batch cultures in 120-mL serum bottles with a working volume of 50 mL. The initial biomass concentration in each bottle was 140 mg/L. The medium comprised (g/L): glucose 0.5, K<sub>2</sub>HPO<sub>4</sub> 0.05, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.3, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2, NH<sub>4</sub>Cl 0.16, EDTA 0.00625 and 1.25 mL/L of a trace element solution. This recipe was adapted from the original version (Raghoebarsing et al. 2006) by removing KHCO<sub>3</sub> to restrict the carbon source to glucose and adding NH<sub>4</sub>Cl to provide a nitrogen source for microbial growth. The trace element solution contained (g/L) the following: EDTA 15, FeSO<sub>4</sub> 5, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.43, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.24, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.99, CuSO<sub>4</sub> 0.25, (NH<sub>4</sub>)<sub>6</sub>MoO<sub>24</sub>·4H<sub>2</sub>O 0.22, NiCl<sub>2</sub>·6H<sub>2</sub>O 0.19, SeO<sub>4</sub> 0.067, H<sub>3</sub>BO<sub>3</sub> 0.014 and Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O 0.050. Oxygen was removed from the test medium by sparging with nitrogen gas. The medium was buffered with 4.1 g/L (20 mM) 3-morpholinopropanesulfonic acid (MOPS) at pH 5.5. This pH is inhibitory to methanogens (Kim et al. 2004) but within the optimum range for fermentative H<sub>2</sub> production (Fang and Liu 2002). The chosen initial glucose concentration of 0.5 g/L was below the threshold substrate inhibition concentration of 1 g/L (Zheng et al. 2008).

### Maintenance of culture

The culture was maintained in 120-mL serum bottles operated with 50 mL working volume and incubated in a 60 °C incubator without any mixing or shaking. The bottles were operated as fed-batch reactors by injecting 0.5 mL of 50 g/L glucose solution through the septum of each bottle after every 24 h. Glucose was rapidly metabolized and exhausted within 24 h, so the peak glucose concentration within the 24-h cycle was always 0.5 g/L. The headspace of each bottle was purged

with N<sub>2</sub> daily after sampling and glucose addition. The headspace was purged by piercing two needles, one for influent and one for effluent, through the septum.

The medium was replaced at 72-h intervals (three glucose additions) by distributing the stirred content of the bottles amongst an equal number of sterilized 50-mL BD Falcon<sup>®</sup> tubes. The tubes were centrifuged at 10,000 rpm for 3 min and the supernatant (old medium) decanted and discarded. The pellet (biomass) from each tube was flushed with fresh medium back to one serum bottle and the working volume of the bottle topped up to 50 mL with fresh medium. Blending the biomass in this fashion during each transfer ensured the same culture was maintained across all the bottles. In each batch, five replicate bottles were maintained at the same time.

In order to disable methanogenic activity, the first three medium replacements were supplemented with 2-bromoethanesulfonate (BES, Sigma-Aldrich, Australia) to achieve a final BES concentration of 4.2 g/L (20 mM). After these three additions, BES was not added with subsequent medium replacements.

### Dynamic profiling of batch fermentation process

In order to understand the dynamics of the fermentation process within the regular fed-batch process, a set of 30 serum bottles were run simultaneously. All bottles were inoculated with 140 mg/L of enriched culture and 0.5 g/L of glucose and incubated at 60 °C. The 30 bottles were split into 10 batches (3 bottles each batch). The liquor from each batch was sampled according to the sampling schedule shown in Table 1. This strategy ensured that the dynamics of the process was captured and that the sampling impact was spread amongst the bottles. The triplicates within each batch also ensured repeatability was measured.

**Table 1** Serial sacrifice sampling design for dynamic profiling of glucose fermentation

Batch	Sampling time since experiment commenced (h)																
	0	1	2	3	4	5	6	7	8	9	10	11	12	18	22	24	
Batch 1	✓										✓						✓
Batch 2			✓									✓					✓
Batch 3				✓									✓				✓
Batch 4					✓									✓			✓
Batch 5						✓										✓	✓
Batch 6							✓										✓
Batch 7								✓									✓
Batch 8									✓								✓
Batch 9										✓							✓
Batch 10	✓																✓

✓ means gas and liquid samples were taken from this batch at this time point

As shown in Table 1, gas and liquid samples were taken at 1-h intervals for the first 12 h and then after 18, 22 and 24 h.

### Isotope feeding experiments

The cultivated biomass was used for isotope feeding experiments where the standard substrate, 0.5 g/L glucose, was supplemented with <sup>13</sup>C-labelled sodium acetate-2-<sup>13</sup>C (labelled on methyl group, 99 atom% <sup>13</sup>C, Sigma-Aldrich<sup>®</sup>, USA). The use of methyl group <sup>13</sup>C-labelled acetate avoided the effects on <sup>13</sup>CO<sub>2</sub> enrichment caused by carboxyl exchange. Sufficient biomass was retained to run 15 bottles, each with an initial biomass concentration of 140 mg/L. Five bottles contained an initial glucose concentration of 0.5 g/L glucose with 50 mg/L <sup>13</sup>C-labelled acetate (CH<sub>3</sub>COO<sup>-</sup> equivalent). Another five bottles contained an initial glucose concentration of 0.5 g/L glucose with no acetate. The remaining five bottles contained 50 mg/L of <sup>13</sup>C-labelled acetate only, but with no glucose. This entire series of 15 bottles was performed in duplicate (30 bottles in total).

All of these bottles were incubated at 60 °C for 2 days, to ensure all reactions were completed when samples were taken. Gas samples from the headspace of the bottles were taken at the beginning and end of the 2-day experiment and analysed for <sup>13</sup>CO<sub>2</sub>/<sup>12</sup>CO<sub>2</sub> ratio.

### Background <sup>13</sup>CO<sub>2</sub> enrichment experiments

In order to account for <sup>13</sup>CO<sub>2</sub> production from the endogenous decay of biomass that had assimilated <sup>13</sup>C from previous feeds of glucose, the cultures from the isotope feeding experiments after 2 days incubation were transferred to identical new bottles by the previously described medium replacement method and fed with 0.5 g/L glucose. The experiments were also allowed to run for 2 days. Gas samples from the headspace of the reactors were taken at the beginning and end of the 2-day experiment to measure the amount of <sup>13</sup>CO<sub>2</sub> released as a result of endogenous decay within the timeframe of an experiment.

### Gas and liquid analyses

Biogas in the headspace of the serum bottles was sampled by piercing the rubber septum with a syringe with a pressure lock (SGE Analytical Science, Australia). H<sub>2</sub> was analysed by gas chromatography (GC) with a method previously described (Zheng et al. 2010). CO<sub>2</sub> and CH<sub>4</sub> were measured with a gas chromatograph (Shimadzu, Japan) equipped with a Porapak Q column and a thermal conductivity detector working at 160 °C with helium as the carrier gas at a flow rate of 20 mL/min. The injection temperature was 110 °C and the oven temperature was 40 °C. Since the experiments were conducted at 60 °C, water vapour condensed on the wall of the gas sampling

syringe, causing more headspace gas to be drawn into the syringe (Kim and Daniels 1991). A correction factor (0.93) calculated by analysing dry and water-saturated standard gas (Zinder and Koch 1984) was applied to all gas analyses.

Liquid samples were also collected by piercing the septum with a syringe. Samples were injected through a 0.22- $\mu\text{m}$  filter (Millipore Corporation, USA) prior to analysis. Glucose, formate, succinate and lactate were analysed with a Shimadzu high-performance liquid chromatograph (HPLC) with a diode array detector (SPD-M10AVP) and a refractive index detector (RID-10A). Compounds were separated with a HPX-87H 300 mm  $\times$  7.8 mm ion exclusion column (Cat. No. 125-0140, BioRad Aminex) by using 0.008 N  $\text{H}_2\text{SO}_4$  as the eluent at a flow rate of 0.4 mL/min. The HPLC was calibrated with glucose, formate, succinate and lactate standard solutions at concentrations of 50, 100, 250 and 500 mg/L.

Volatile fatty acids (VFAs) and alcohols were analysed with an Agilent Technologies 7890A gas chromatograph (GC) with a flame ionization detector (FID). Calibration was carried out using standard solutions made up of six VFAs (acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and hexanoate) and two alcohols (ethanol and butanol) with concentrations of 20, 50, 100 and 500 mg/L.

Qualitative polyhydroxyalkanoate (PHA) analysis was conducted by using the Fourier transform infrared (FT-IR) spectroscopy method (Arcos-Hernandez et al. 2010).

The biomass concentration was assumed to be equal to the suspended volatile solid, measured by using the standard method (Clesceri et al. 1998).

To determine the chemical formula of biomass, biomass was pelleted and washed in reverse osmosis (RO) water before sending out for analysis. Fifty milliliters of slurry sample was centrifuged in a 50-mL BD Falcon<sup>®</sup> tube at 4000 RPM for 10 min. The supernatant was discarded and the pellet was resuspended in 50 mL RO water. The sample was then centrifuged again at 4000 RPM for 10 min. The washed cell pellet was then freeze dried (to remove free water), weighted and analysed for carbon, hydrogen and nitrogen by a TruSpec<sup>®</sup> CHN analyser (LECO Australia Pty. Ltd., Australia) according to the standard method (Rayment and Higginson 1992). The balancing weight of those three measured compounds is assumed to be oxygen.

### Isotope analysis

The  $^{13}\text{C}/^{12}\text{C}$  ratio in the gas samples was analysed by a continuous-flow stable isotope ratio mass spectrometer (CF-IRMS) coupled with a MicroMass manual injection micro gas box (Isoprime Ltd., UK). Laboratory standard calcite ANU-M2, which had previously been calibrated against IAEA standard NBS18, was also analysed in the same way as the sample gases. Each sample was analysed in triplicate. Results are expressed by using the standard  $\delta$  notation and units of per

mille (thousand) relative to the Vienna Pee-Dee Belemnite (PDB) carbonate standard as follows (Kendall and McDonnell 1998):

$$\delta^{13}\text{C}_{PDB} = \left( \frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C}_{\text{standard}}} - 1 \right) \cdot 1000 \quad (2)$$

where

$^{13}\text{C}/^{12}\text{C}_{\text{sample}}$  is the ratio of  $^{13}\text{CO}_2$  to  $^{12}\text{CO}_2$  in the gas sample  
 $^{13}\text{C}/^{12}\text{C}_{\text{standard}}$  is the ratio of  $^{13}\text{CO}_2$  to  $^{12}\text{CO}_2$  in the standard, =0.0112 (Kendall and McDonnell 1998)

The accuracy of measurement was  $\pm 0.2 \delta^{13}\text{C}_{PDB}$ .

### Calculation of cumulative $\text{H}_2$ yield

Cumulative  $\text{H}_2$  yields ( $Y_{\text{H}_2, \text{cumu}}$ ) were calculated as

$$Y_{\text{H}_2, \text{cumu}} = \frac{H_{2, \text{cummu}, t}}{\text{glu}_{\text{cumu}, t}} \quad (3)$$

where

$H_{2, \text{cumu}, t}$  is the cumulative  $\text{H}_2$  production at time  $t$  (mol)  
 $\text{glu}_{\text{cumu}, t}$  is the cumulative glucose consumption at time  $t$  (mol)

### Calculation of the theoretical fermentation $\text{H}_2$ production

The theoretical fermentation  $\text{H}_2$  yield was calculated as

$$H_{2, \text{ferm}} = \sum_i \text{VFA}_i \cdot Y_{\text{VFA}_i / \text{H}_2} \quad (4)$$

where

$H_{2, \text{ferm}}$  is the theoretical fermentative  $\text{H}_2$  yield (mol)  
 $\text{VFA}_i$  is the mass of  $\text{VFA}_i$  (mol)  
 $Y_{\text{VFA}_i / \text{H}_2}$  is the theoretical molar  $\text{H}_2$  yield associated with the production of  $\text{VFA}_i$ , e.g.  $Y_{\text{VFA}_i / \text{H}_2} = 2, -2$  and  $2$  for acetic, propionic and butyric acids respectively (mol  $\text{H}_2$ /mol  $\text{VFA}_i$ )

The percentage of excess hydrogen production was calculated as

$$\% \text{ excess } \text{H}_2 \text{ production} = \frac{H_{2, \text{meas}} - H_{2, \text{ferm}}}{H_{2, \text{ferm}}} \cdot 100\% \quad (5)$$

where

$H_{2, \text{means}}$  is the measured  $\text{H}_2$  production (mol)

## Calculation of the dissolved CO<sub>2</sub> production

Calculation of the total CO<sub>2</sub> production must take into account dissolved CO<sub>2</sub>. On the basis that CO<sub>2</sub> had equilibrated between the liquid and gas phases, the dissolved CO<sub>2</sub> concentration was calculated by using Henry's law (Eq. 7).

$$C_{\text{CO}_2} = p_{\text{CO}_2} \cdot k_{\text{H}} \quad (6)$$

where

- $C_{\text{CO}_2}$  is the dissolved CO<sub>2</sub> concentration (mol/L)  
 $p_{\text{CO}_2}$  is the partial pressure of CO<sub>2</sub> in the gas phase above the liquid (atm)  
 $k_{\text{H}}$  is the Henry's law constant (mol/L·atm), =0.015 at 60 °C

## Microbial Community analysis

### DNA Extraction

Genomic DNA was extracted from phosphate-buffered saline (PBS)-washed fresh biomass, which was taken from serum bottles at the end of 3-day cycles, using the FastDNA spin kit for soil (Q-Bio gene, Australia) and Fastprep beadbeating machine (Bio101, USA) according to the manufacturer's instructions. The extracted DNA was electrophoresed in 1 % agarose to check for shearing (Sambrook et al. 1989), and the concentration was verified using NanoDrop ND-1000 (Thermo Fisher Scientific, USA) before storing at -20 °C. The purity of DNA was assessed by using the A260/A280 and A260/230 absorbance ratios.

### Pyrotag amplification

The 16S ribosomal RNA (rRNA) genes were sequenced by using universal primer set 926F (5'-AAACTYAAK GAATTGACGG-3') and 1492R (5'-GGTACCTTGT TACGACTT-3') and *Bacteria* and *Archaeal* primer set 1114F (5'-GCAACGAGCGCAACC-3') and 1392R (5'-ACGGGCGGTGTGTRC-3'). Primer sequences were modified by the addition of Roche 454 adaptor 1 or 2 sequences and unique 5-bp barcodes at the 5' end of the primer (sequences not shown) (Engelbrektson et al. 2010; Kunin et al. 2010).

After extraction, the original extracted DNA was lyophilised using Savant SpeedVac Concentrator SVC100H (Thermo Fisher Scientific, USA) and submitted to the U.S. Department of Energy Joint Genome Institute (DOE JGI) for 16S rRNA gene pyrotag sequencing on the Genome Sequencer FLX Titanium platform (Roche, USA).

### Pyrotag sequencing and bio-informatics analysis

Pyrotag sequences were processed as described by Engelbrektson et al. (2010). The barcodes and amplicon primer sequences were removed and the ends of the reads were trimmed with LUCY (Li and Chou 2004) based on their quality values. The reads were clustered at the 97 % similarity level using the QIIME (Caporaso et al. 2010). Cluster representative sequences were compared to the NCBI database using BLAST to identify their taxonomy (Tamura et al. 2011). Searches for the closest matches for the gene sequences were carried out using the BLAST database (Altschul et al. 1990).

Sequences of the operational taxonomic units (OTUs) were submitted to GenBank with accession numbers of KU363984, KU363985 and KU363986.

### Fluorescence in situ hybridisation

A fluorescence in situ hybridisation (FISH) probe specific for the *Thermoanaerobacterium thermosaccharolyticum* species, developed and tested by O-Thong et al. (2008a), was used to confirm the relative abundance of this group within the enrichment culture. An additional probe used in FISH with the fixed biomass was EUBMIX (Amann et al. 1990; Daims et al. 1999). The probes were synthesized and labelled at the 5' end with the sulfoindocyanine dyes Cy3 and Cy5 (Thermohybrid Interactiva, Ulm, Germany).

FISH was carried out with 35 % formamide concentration as determined by O-Thong et al. (2008a) as the optimum for the Tmbthsacc184 probe. Samples of biomass from the enriched culture were fixed in both ethanol (1:1) and paraformaldehyde (3:1) (Manz et al. 1992). FISH was carried out according to previously described methods (Amann 1995). The abundance of the *T. thermosaccharolyticum* cells was assessed as the percentage of target cells relative to the number of cells binding the universal bacterial probe mix (Daims et al. 1999).

For analysis of the FISH, samples were examined with a Zeiss LSM 510 META confocal laser-scanning microscope (CLSM) using a Nikon ×63 oil immersion objective. Images were captured using the LSM 510 image acquisition software package (AIM 4.2). In order to avoid autofluorescence from unstained organisms (green emission), the green channel was not collected for images used for quantification. Quantification of the abundance of the bacteria targeted by the specific probe relative to those targeted by the EUBMIX probes was carried out in the Daime image analysis software (Daims et al. 2006). Relative abundance was measured as the ratio of the area of fluorescence conferred by the CY3-labelled EUBMIX probe compared to that emitted by the Cy5-labelled Tbmthsacc184 probe. Two separate samples (A and B), both collected at the same time, were used for the collection of FISH images to allow assessment of sample variation. In total,

61 images from the A sample and 53 images from the B sample were collected for image analysis. The error bars provided in the figures represent the 95 % confidence interval for that data set.

## Results

### Culture enrichment

The culture was maintained for 2 years by following the culture enrichment method described in the ‘Materials and methods’ section before being used in this study. This enrichment/maintenance process has resulted in a stable and reproducible trend in glucose consumption and H<sub>2</sub> production. A snapshot of 12 days culture performance is shown in Fig. 1. These trends were recorded over 12 days from three successive enrichment cycles in five replicate bottles, in which the normal 3-day cycle was extended to the addition of a fourth pulse before replacing the medium, and the headspace was not purged until the end of the cycle. These modifications were made to illustrate the distinct inhibition threshold of the culture. Each of the first three glucose pulses was completely consumed within 24 h, with highly repeatable H<sub>2</sub> and VFA yields for each pulse, while degradation of the fourth pulse was inhibited. H<sub>2</sub> reached a partial pressure of  $0.34 \pm 0.01$  atm by the end of the third glucose addition. The small error bars illustrate the repeatability of the 4-day cycle.

Although the onset of inhibition appears sudden, deterioration in activity during the first three daily feeds will have been masked because glucose was always exhausted when measurements were made. Deterioration only affected H<sub>2</sub> yield when it took longer than 24 h to utilize the glucose feed, which repeatedly occurred after the fourth feed of glucose (day 4 in

Fig. 1). Daily H<sub>2</sub> yields were therefore routinely maintained by replacing media after three feeds of glucose.

### Metabolic products

In the 3-day feeding cycles, H<sub>2</sub> yield diminished slightly as fermentation products accumulated between each medium and headspace replacement. Therefore, H<sub>2</sub> yield in response to the initial glucose addition (day 1) was regarded to be the uninhibited yield. The H<sub>2</sub> production in response to the initial glucose additions was  $2.7 \pm 0.1$  mol H<sub>2</sub>/mol glucose.

Acetate and butyrate were the main liquid-phase metabolic products. Together with H<sub>2</sub>, they accounted for  $86 \pm 3.6$  % of the product COD. Ethanol and butanol were also detected in the liquid-phase products, which accounted for less than 5 % of the product COD. Biomass yield measurement showed that 6.8 % of the total COD was assimilated into biomass, whose chemical formula was confirmed by the CHN analysis as CH<sub>2.37</sub>O<sub>0.43</sub>N<sub>0.22</sub>.

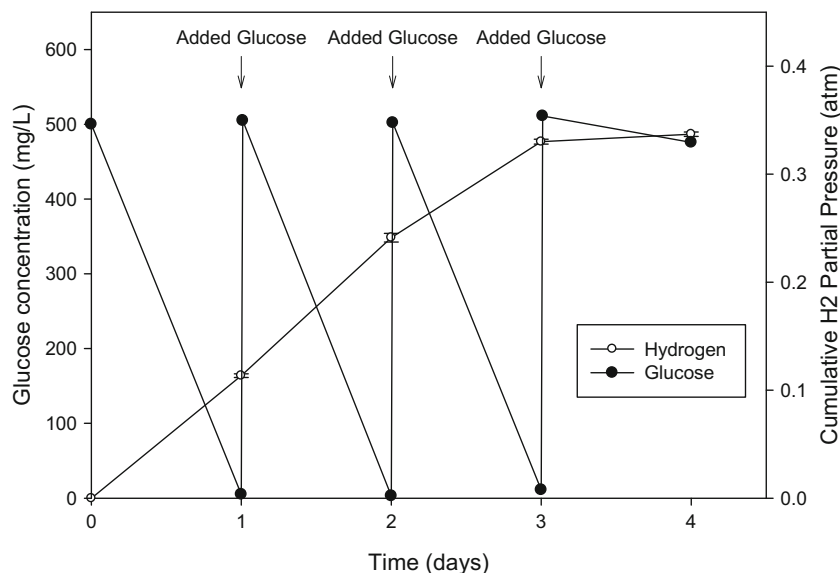
PHA analysis showed that no PHA was accumulated during the fermentation processes as they were all below the detection limit of the measurement, which is 0.02 mg PHA biomass. Similarly, formate, lactate and succinate in the samples were always below the detection limit of 0.1 mg/L.

All the above products closed the COD balance to within 2 %, indicating that the measurements were accurate (Table 2).

### Fermentation metabolites’ dynamic profiles

In the separate profiling experiments that sampled along the fermentation process, analysis showed that glucose started to be consumed 2 h after experiments commenced (Fig. 2). An exponential consumption stage was observed from 8 till 12 h, when it was completely consumed. As a

**Fig. 1** Glucose and H<sub>2</sub> profiles from a fed-batch experiment starting with fresh media where liquid and gaseous products were allowed to accumulate for 4 days (four glucose additions). Error bars are standard deviations of 15 bottles



**Table 2** Average COD balance from the first day of each cycle within the 12-day snapshot period, based on 15 bottles fed with glucose. All units are in milligrams·COD except H<sub>2</sub> yield which is expressed as moles

H<sub>2</sub>/moles glucose. Carbon balances for all the batches were within 97 % (data not shown). Formate, lactate, succinate, propionate and valerate were not detected in any of the batches

	Glucose consumed	A.A.	B.A.	ETOH	BTOH	H <sub>2</sub>	Biomass	Balance	H <sub>2</sub> yield
Average	-27.83	5.28	12.83	0.89	0.44	5.94	1.89	-0.55	2.68
Percentage (%)	-100	18.96	46.10	3.20	1.57	21.36	6.80	-1.97	
Standard deviation ( <i>n</i> = 15)	0.00	0.29	0.38	0.10	0.05	0.14	0.05	0.36	0.06

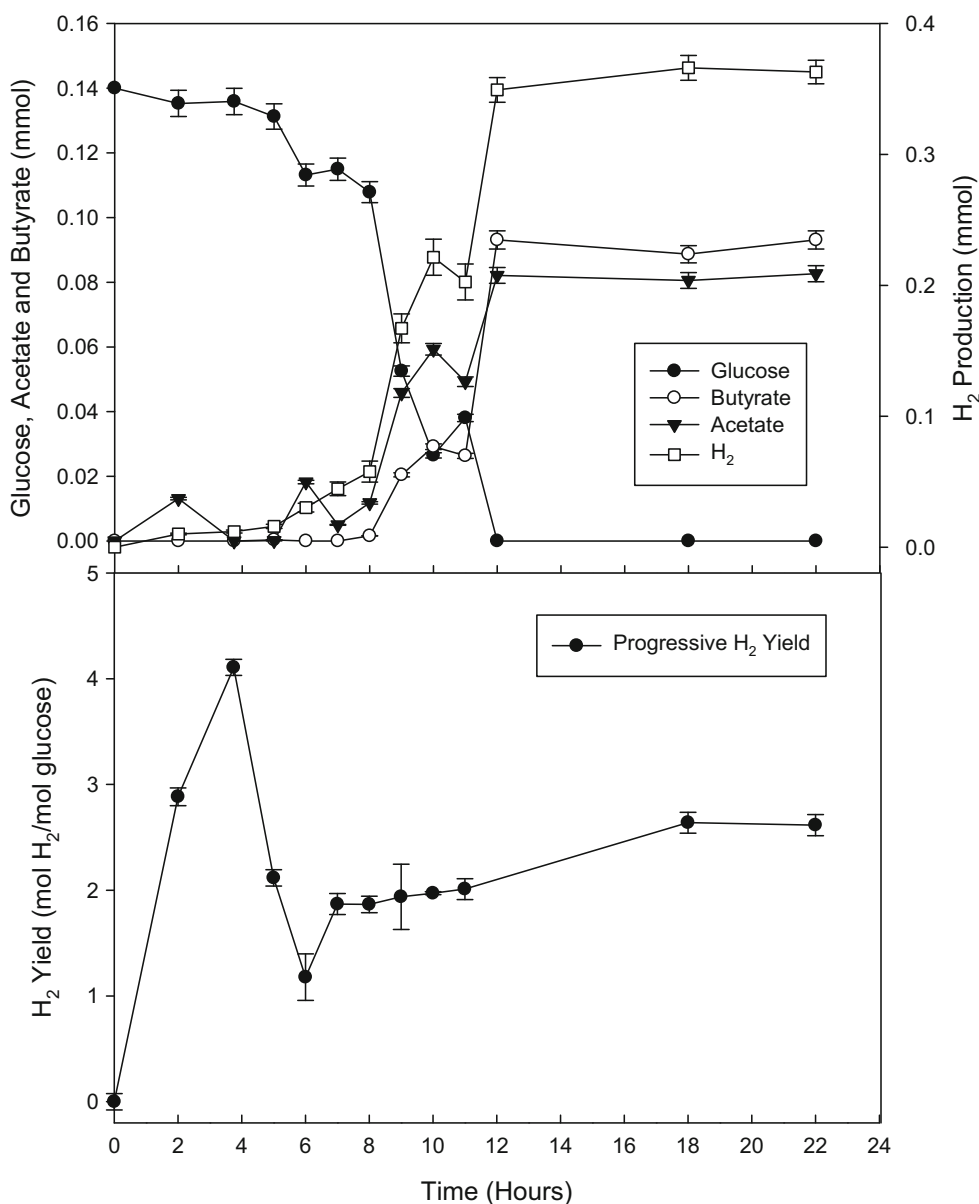
A.A. acetic acid, B.A. butyric acid, ETOH ethanol, BTOH butanol

result, H<sub>2</sub> and acetate production started to be observed from 2 h as well. However, butyrate production only started to be observed after 8 h. Following glucose consumption, the metabolite productions experienced an exponential growth in the same 8-12-h period. After 12 h,

all metabolite yields ceased except H<sub>2</sub>, which slowly stopped around 18 h.

The progressive H<sub>2</sub> yields, which represent the yield at different fermentation stages, showed that in the initial 4 h H<sub>2</sub> yield increased sharply to 4.11 ± 0.2 mol H<sub>2</sub>/mol glucose consumed,

**Fig. 2** Dynamic profiles of major fermentation products and progressive H<sub>2</sub> yields at different fermentation stages



which is followed by a sharp decrease to  $1.18 \pm 0.2$  mol H<sub>2</sub>/mol glucose consumed at 6 h. Then, the yield slowly increased to its final  $2.61 \pm 0.1$  mol H<sub>2</sub>/mol glucose at 22 h.

### Stable isotope-labelled acetate feeding experiments

Measured  $\delta^{13}\text{C}_{\text{PDB}}$  values of the bottles containing glucose and <sup>13</sup>C-labelled acetate are shown in Fig. 3. The higher the value of  $\delta^{13}\text{C}_{\text{PDB}}$ , the more labelled acetate was consumed in the relevant reactor.  $\delta^{13}\text{C}_{\text{PDB}}$  for bottles fed with glucose but no labelled acetate was  $-18.5 \pm 0.4$  ‰. The biomass decay experiments showed that no <sup>13</sup>CO<sub>2</sub> enrichment was caused by the biomass decay effect (data not shown). The average value of  $\delta^{13}\text{C}_{\text{PDB}}$  in the bottles containing both glucose and <sup>13</sup>C-labelled acetate was  $-6.4 \pm 8.8$  ‰.

### Microbial community

From the pyrosequencing analysis, 6346 sequences were sequenced. Sequences were grouped into operational taxonomic units (OTU) when sequences were  $\geq 98$  % similar, which were also very similar to each other and are, most likely, strains of the same species. All sequences were clustered into three OTUs. Sequences of the OTUs were submitted to GenBank with accession numbers of *KU363984*, *KU363985* and *KU363986*. With the detection accuracy of up to genus level, the pyrosequencing analysis showed that the OTU sequences, which accounted for over 99.4 % of the overall sequences, belong to the genus of *Thermoanaerobacterium* and had high identity to the previously described bacterium, *T. thermosa ccharolyticum*.

Examination of the ProbeCheck database (Loy et al. 2008) confirmed the existence of a FISH probe for *T. thermosa ccharolyticum*. Due to the very high similarity of the recovered

sequences to the isolates and existing sequences and to each other, it was not possible to design new probes to distinguish the organisms enriched in this case from existing isolates or from each other. FISH with the previously published probe designed to target the *T. thermosaccharolyticum* species (O-Thong et al. 2008a) confirmed the pyrosequencing results (Fig. 4).

The hydrogen yield and the yields of other products observed from this study were similar to those observed in another study conducted using pure cultures of *T. thermosaccharolyticum* (O-Thong et al. 2008b).

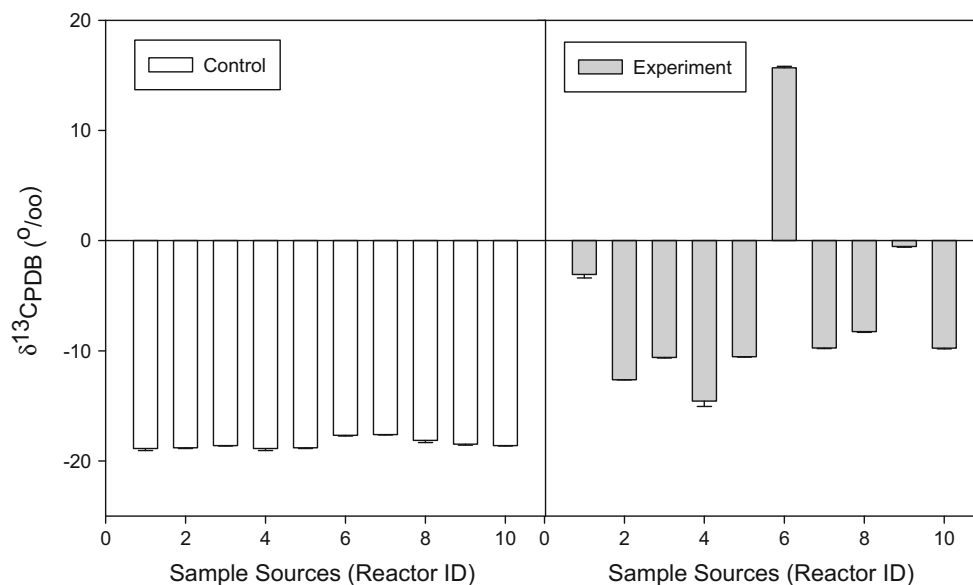
## Discussion

### H<sub>2</sub> fermentation metabolic pathway

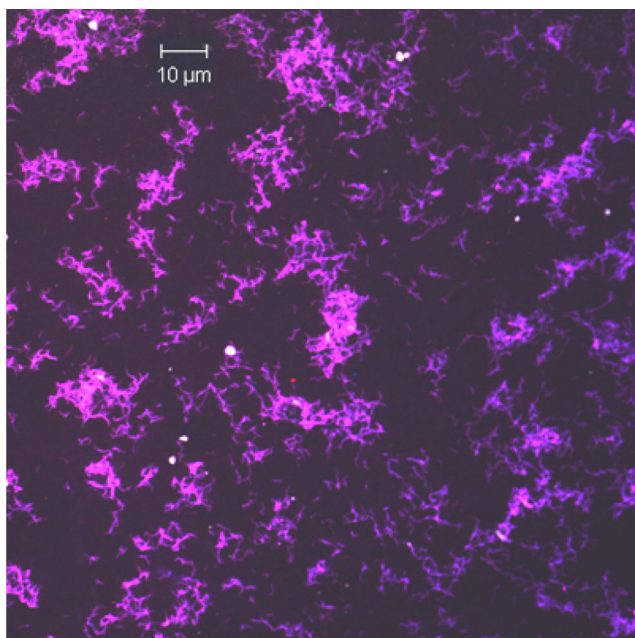
The experiments showed that the amount of H<sub>2</sub> production is constant. This is expected given that the initial amount of biomass and glucose added to each batch experiment was constant. To better understand the H<sub>2</sub>-producing pathways, a detailed mass balance was conducted by calculating the theoretical H<sub>2</sub> yield from the well-reported acetate and butyrate fermentation pathways and comparing it with the measured H<sub>2</sub> yield (Table 3). The mass balance showed that the measured H<sub>2</sub> production exceeded the theoretical fermentative H<sub>2</sub> yield, H<sub>2,ferm</sub>, by an average of 14 % (based on Eq. 5).

The same mass balance approach has previously been used by Kotsopoulos et al. (2006) in their H<sub>2</sub> fermentation study and observed excess H<sub>2</sub> yield, indicating this might be a commonly existing but undercovered phenomenon. A further literature results review by using the same method was conducted and the Table 4 was generated (Kotsopoulos et al. 2006) (O-Thong et al. 2008b) (Lay 2000). It shows that excess H<sub>2</sub> was produced in most of the studies, confirming the wide

**Fig. 3**  $\delta^{13}\text{C}_{\text{PDB}}$  values of CO<sub>2</sub> for experiments fed with glucose and <sup>13</sup>C-acetate and the corresponding control experiments (*blank*) fed with glucose only. Error bars are standard deviations of duplicated experiments







**Fig. 4** FISH image collected with EUBMIX probe labelled with CY3 (red) and Tbmthsacc184 probe labelled with CY5 (blue); thus, the overlap of these two probes (purple) are *Thermoanaerobacterium thermosaccharolyticum* cells

existence of excess  $H_2$  yield. It should also be noted that, by comparing those studies, the extents of excess  $H_2$  productions were not showing a clear relation to the operating conditions, e.g. operating temperatures.

On the other hand, the dynamic profiling of fermentation metabolites indicated that the butyrate formation pathway was the dominant  $H_2$ -yielding pathway at the beginning of the process (0–4 h). The measured system  $H_2$  yield has climbed up to over 4.0 mol  $H_2$ /mol glucose in the same period. However, no acetate was detected in the same period, indicating it is either not produced or consumed at a rate that is higher than the production rate.

While these mass balances and dynamic profiling provided compelling evidence that higher  $H_2$ -yielding metabolic pathways occurred, e.g. acetate oxidation, more direct evidence of acetate oxidation in the absence of syntrophic  $H_2$  sinks such as methanogens has not been published. Therefore, whether acetate oxidation was the reason for excess  $H_2$  production was not clear.

To investigate this, a  $^{13}C$ -labelled acetate co-feeding experiment was conducted. Using the measured enrichment of  $^{13}CO_2$  in the gas-phase production as the evidence, no significant acetate consumption was observed in the co-feeding experiment, indicating that acetate oxidation either did not happen in the system or is not obvious when it occurs in conjunction with glucose fermentation.

It is worthy to note, similar to the glucose feeding experiment, in the  $^{13}C$ -labelled acetate and glucose co-feeding experiment, measured  $H_2$  was also in excess of the theoretical fermentation yield by around 14 % (data not shown).

The fact that CHN analysis showed a biomass chemical formula of  $CH_{2.37}O_{0.43}N_{0.22}$ , which is a product that is more reduced than glucose suggesting biomass is a sink of reducing equivalents (i.e.  $H_2$ ). Combining with the observed over 4.0 mol  $H_2$ /mol glucose  $H_2$  yield when only the butyrate production pathway was detected, it is suggested that metabolic pathways that have not been reported previously have taken place in this system, especially in the initial stage. Such pathway might not be detected if it only occurs at a certain stage of the fermentation process.

## $H_2$ concentration effects

The thermodynamics of a biochemical reaction varies when the reaction condition changes (e.g. dissolved  $H_2$  concentration); it is therefore hypothesised that the final excess  $H_2$  yield and declining of  $H_2$  yield after 4 h were partly due to dissolved  $H_2$  concentration changes.

In a similar batch system study conducted by Zheng et al. (2010), who monitored the oversaturation ratios (dissolved  $H_2$  concentration to headspace  $H_2$  concentration ratio) in the system, a bottom-up V-shape ( ) pattern of oversaturation ratio change was also observed. Interestingly, the observed  $H_2$  oversaturation started at around 5 h and peaked at 6 h, after which it started to drop. In this study, the peak  $H_2$  yield was also observed at 4 h and started to drop immediately afterward. Combining the observed  $H_2$  oversaturation by Zheng et al. (2010), this is when the thermodynamic favourable conditions would be changed significantly once the  $H_2$  has been produced especially in a non-stirred system, where  $H_2$  can be

**Table 3** Comparison of product yields with  $H_{2, \text{ferm}}$  based on 15 bottles fed with glucose

	Measured product yields from 0.14 mmol of glucose ( $\approx 50$ ml of 0.5 g/L glucose)			$H_{2, \text{ferm}}$			H2 production in excess of fermentative yield
	Acetate [mmol AA]	Butyrate [mmol BA]	$H_2$ [mmol $H_2$ ]	Acetate [mmol $H_2$ ]	Butyrate [mmol $H_2$ ]	Sub-total [mmol $H_2$ ]	[mmol $H_2$ ]
Average	0.09	0.08	0.37	0.17	0.16	0.33	0.05 (14 %)
Standard deviation ( $n = 15$ )	0.01	0.00	0.01	0.01	0.01	0.01	0.01

**Table 4** Summary of H<sub>2</sub> production-related metabolic products from various fermentation studies and comparison between their theoretical associated H<sub>2</sub> production and measured H<sub>2</sub> production

	Study conditions			Liquid analysis			H <sub>2</sub> production		
		P.A.	B.A.	A.A.	B.A.	Measured	Calculated	ΔH <sub>2</sub>	ΔH <sub>2</sub> (%)
		mmol/day	mmol/day						
70 °C glucose-fed UASB (Kotsopoulos et al. 2006)	R1	1.38 mmol/day	0.87 mmol/day	0.87 mmol/day	11.15 mmol/day	4.48 mmol/day	6.67 mmol/day	149	
	R2	3.73 mmol/day	0.55 mmol/day	0.55 mmol/day	5.93 mmol/day	8.55 mmol/day	-2.62 mmol/day	-31	
	R3	1.67 mmol/day	0.79 mmol/day	0.79 mmol/day	9.33 mmol/day	4.83 mmol/day	4.50 mmol/day	93	
60 °C carbohydrate-fed-batch (O-Thong et al. 2008b)	Glucose	6.73 mmol/L	-	1.46 mmol/L	39.2 mmol/L	17 mmol	22.2 mmol	130	
	Sucrose	17.78 mmol/L	-	0.67 mmol/L	80.4 mmol/L	36.9 mmol	43.5 mmol	118	
37 °C starch-fed Chemostat CSTR (Lay 2000)	Starch	11.68 mmol/L	-	0.90 mmol/L	70.7 mmol/L	25.8 mmol	44.9 mmol	174	
	15 h HRT	4.2 mmol/L	6.8 mmol/L	5.1 mmol/L	6.55 mmol/h	2.36 mmol/h	4.19 mmol/L	178	
	20 h HRT	10 mmol/L	1.4 mmol/L	12.5 mmol/L	7.63 mmol/L	6.54 mmol/L	1.09 mmol/L	17	
60 °C carbohydrate-fed batch (Ren et al. 2008)	25 h HRT	6.7 mmol/L	8.4 mmol/L	5.5 mmol/L	4.92 mmol/L	1.92 mmol/L	3.0 mmol/L	156	
	Glucose	33.3 mmol/L	-	15.9 mmol/L	193.5 mmol/L	98.4 mmol/L	95.1 mmol/L	97	

- not presented in this study

oversaturated up to 180 times in the liquid phase (Kraemer and Bagley 2006; Zheng et al. 2010).

As previously reported by Thauer et al. (1977), the most observed 2.0 mol H<sub>2</sub>/mol glucose yield was the result of the ferredoxin (Fd) reduction reaction, which can spontaneously happen under a normal H<sub>2</sub>-accumulating environment. Additional H<sub>2</sub> yield can be expected from pathways such as NADH to NAD<sup>+</sup> that is coupled with the H<sup>+</sup> to H<sub>2</sub> reaction. However, this reaction is only thermodynamically favourable when the dissolved H<sub>2</sub> concentration is low (Thauer et al. 1977). Zheng et al. (2015) have also observed this dissolved H<sub>2</sub> concentration-controlled metabolic pathway shifts in their perfusion system, confirming low dissolved H<sub>2</sub> concentration has enhanced the H<sub>2</sub> yield as a result of the created favourable thermodynamic conditions.

The consistent observation of the H<sub>2</sub> oversaturation pattern and H<sub>2</sub> yield change pattern in two separate batch systems suggested that lower H<sub>2</sub> concentration in the initial fermentation stage was likely the reason for the observed higher H<sub>2</sub> yield.

In summary, a highly enriched culture of *T. thermosa ccharalyticum* has been cultured from a methanogenic anaerobic digester and has given a stable H<sub>2</sub> yield of 2.7 ± 0.1 mol H<sub>2</sub>/mol glucose for over 2 years. The comprehensive metabolic pathway study on this stable culture has indicated that a better thermodynamic condition has been created at the initial fermentation stage, and therefore, a higher H<sub>2</sub> yield was observed. This result suggested further studying of the early fermentation stage is a potential direction for promoting H<sub>2</sub> yield in a mixed culture study and comprehensive product analysis is essential to understanding fermentation processes in depth.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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