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Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation

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Abstract

The biochemical hydrogen potential (BHP) tests were conducted to investigate the metabolism of glucose fermentation and hydrogen production performance of four Clostridial species, including *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9. Batch experiments showed that all the tested strains fermented glucose, reduced medium pH from 7.2 to a value between 4.6 and 5.0, and produced butyrate (0.37–0.67 mmol/mmol-glucose) and acetate (0.34–0.42 mmol/mmol-glucose) as primary soluble metabolites. Meanwhile, a significant amount of hydrogen gas was produced accompanied with glucose degradation and acid production. Among the strains examined, *C. beijerinckii* L9 had the highest hydrogen production yield of 2.81 mmol/mmol-glucose. A kinetic model was developed to evaluate the metabolism of glucose fermentation of those *Clostridium* species in the batch cultures. The model, in general, was able to accurately describe the profile of glucose degradation as well as production of biomass, butyrate, acetate, ethanol, and hydrogen observed in the batch tests. In the glucose re-feeding experiments, the *C. tyrobutyricum* FYa102 and *C. beijerinckii* L9 isolates fermented additional glucose during re-feeding tests, producing a substantial amount of hydrogen. In contrast, *C. butyricum* ATCC19398 was unable to produce more hydrogen despite additional supply of glucose, presumably due to the metabolic shift from acetate/butyrate to lactate/ethanol production.

Keywords: Biological hydrogen production; *Clostridium acetobutylicum*; *C. butyricum*; *C. tyrobutyricum*; *C. beijerinckii*; Kinetic model

1. Introduction

The consequences of extensive uses of fossil fuels have raised two severe problems including the issues of depletion of energy resources in the near future and global climate change. Considering the energy security and the global environment, there is an urgent need in developing a clean and renewable energy source. Hydrogen is a clean energy source, generating only water when it burns. In addition, it can be produced from potential renewable raw materials such as organic wastes. Several processes may be applied to produce hydrogen including electrolysis of water, thermocatalytic reformation of hydrogen-rich organic compounds, and biological processes. Fermentative production of hydrogen is an exciting area of technology development that offers a potential means to produce hydrogen from a variety of renewable resources. Through fermentation processes, hydrogen gas can be produced directly from high concentrations of renewable substrates such as sugars or even wastewaters. The theoretical yield of hydrogen from glucose fermentation can be estimated by a known metabolic pathway [1], giving a maximum yield of 4 mol hydrogen per mol of glucose when acetic acid is produced as the terminal metabolite.

Many studies reported that hydrogen can be produced from wastewater or solid waste by mixed cultures in batch or chemostat reactors [2–5], but with a wide fluctuation in hydrogen production performance. The relatively unstable and unpredictable biological hydrogen production processes are primarily dependent on fermentation conditions such as pH [2,6,7], hydraulic or solid retention time [8,9], hydrogen gas partial pressure

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Nomenclature

S_{Glu}	glucose concentration
S_{HAc}	acetate concentration
S_{HBu}	butyrate concentration
S_{Eth}	ethanol concentration
S_{H_2}	hydrogen gas production in volume
f_{HAc}	yield coefficient of acetate production from glucose
f_{HBu}	yield coefficient of butyrate production from glucose
f_{Eth}	yield coefficient of ethanol production from glucose
f_{H_2}	yield coefficient of hydrogen production from glucose
Y	biomass yield coefficient
X	biomass concentration
$Y_{Biomass}$	product yield of biomass
Y_{H_2}	product yield of hydrogen gas
Y_{CO_2}	product yield of carbon dioxide
Y_{HLa}	product yield of lactate
S_{HFo}	product yield of formate
Y_{HAc}	product yield of acetate
Y_{HPr}	product yield of propionate
Y_{HBu}	product yield of butyrate
$Y_{Ethanol}$	product yield of ethanol
$Y_{Propanol}$	product yield of propanol
$Y_{Butanol}$	product yield of butanol
q_G^{max}	maximum specific glucose consumption rate
K_{Glu}	Monod half-saturation constant

[10,11], concentration of acids [7,12], presence of methane-producing microorganisms [13], and microbial community of hydrogen-producing bacteria [14]. Recent reports pointed out that *Clostridium* species were the dominant microorganisms in anaerobic hydrogen fermentation processes [14–18], but their contributions in hydrogen production have not yet been identified quantitatively.

Clostridial species are known as classical acid producers and usually ferment glucose to butyrate, acetate, carbon dioxide, and molecular hydrogen [1,19]. Previous studies regarding clostridial fermentation of pure cultures have extensively focused on solvent production [1,20], while little is known about their ability to produce hydrogen. In this study, the biochemical hydrogen potential (BHP) tests [21] were conducted to assess the metabolism of glucose fermentation and hydrogen production performance of *C. butyricum* ATCC19398 obtained from the American Type Culture Collection (ATCC) and three *Clostridium* species (namely, *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9) isolated from practical hydrogen fermentation processes. In addition, a mathematical model was developed to quantitatively describe and evaluate the metabolism of glucose fermentation and product formation from batch cultures of the *Clostridium* species.

2. Materials and methods

2.1. The microorganisms

The pure culture of *C. butyricum* ATCC19398 was obtained from the ATCC. The isolates of *C. acetobutylicum* M121 and *C. tyrobutyricum* FYa102 were obtained from Dr. I.C. Tseng of Department of Life Science at the National Cheng Kung University, while the *C. beijerinckii* L9 strain was provided by Dr. C.C. Huang of Department of Life Science at the National Chung Hsing University. The pure strains of *C. acetobutylicum* M121 and *C. tyrobutyricum* FYa102 were isolated from a lab-scale continuous flow anaerobic hydrogen fermentor (2 l) fed with glucose and peptone. These strains were identified based on 16S rDNA sequencing analysis. The pure culture of *C. beijerinckii* L9 was isolated from a pilot-scale semi-continuous flow anaerobic hydrogen fermentor (1000 l) fed with wasted yeast residues from a beer fermentation plant and its identity was also confirmed by 16S rDNA sequencing analysis. Prior to the hydrogen production experiments, the isolates were precultured using the same medium as that used in the BHP tests.

2.2. Biochemical hydrogen potential (BHP) test

The BHP test employed in this study was a modified version of biochemical methane potential (BMP) test developed by Owen et al. [21]. The test for each *Clostridium* species was carried out in a series of 120 ml serum bottles. To each bottle with a liquid working volume of 80 ml, 3000 mg/l of glucose, and 2000 mg/l of peptone were added as fermentation substrates. In addition, each bottle contained the following chemicals as growth nutrients (in mg/l): resazurin, 0.175 ; $CaCl_2 \cdot 6H_2O$, 32.32; $MgCl_2 \cdot 6H_2O$, 232.26; KCl, 167.81; $MnCl_2 \cdot 4H_2O$, 63.87; $CoCl_2 \cdot 6H_2O$, 3.87; H_3BO_3, 0.74; $CuCl \cdot 2H_2O$, 0.35; $Na_2MoO_4 \cdot 2H_2O$, 0.33; $ZnCl_2$, 0.27; $FeCl_2 \cdot 4H_2O$, 10.62; sodium thioglycolate, 217.35; KH_2PO_4, 119. The medium pH was adjusted to 7.2 using 3N KOH and 3N HCl. The bottles were capped tightly with rubber septum stoppers, flushed with oxygen free nitrogen gas, and then sterilized in an autoclave. The pure culture was then inoculated into the bottles and was incubated in an orbital shaker with a rotational rate of 120 rpm and a temperature of $35 \pm 1\,^{\circ}C$. For each pure culture studied, BHP tests were conducted at least in duplicated experiments, and some of them were even repeated three to six times. The measured data of the BHP tests served the purpose of parameter estimation of the mathematical model.

2.3. Analytical methods

The composition of biogas in the headspace was analyzed using a gas chromatograph (China GC 8900, Taipei, Taiwan)

equipped with a thermal conductivity detector (TCD). A 2 m stainless column packed with Hayesep Q (60/80 mesh) was installed in a 60 °C oven. The operational temperatures of the injection port, the oven, and the detector were all set at 60 °C. Nitrogen was used as the carrier gas at a flow rate of 15 ml/min. The alcohols were measured using a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan) equipped with a flame ionization detector (FID) and a Supelco column (column # 20476-01F). The operational temperatures of the injection port and the detector were set at 220, and 210 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Concentrations of volatile fatty acids (VFAs) were determined using an ion chromatography (Dionex DX-120, California, USA) equipped with an anion IonPac ICE-ASI column, an AMMA-ICE II suppressor, and a conductivity detector. One mM of heptaflourobatyic acid was used as the eluent at a flow rate of 0.8 ml/min. Five mM of tetrabulammonium hydroxide was used as the regenerant. The carbohydrate was analyzed using the phenol–sulfuric acid method [22]. The pH, NH_4^+–N, chemical oxygen demand (COD) and volatile suspended solids (VSS) were measured according to standard methods [23].

2.4. Mathematical model simulation

In addition to biomass growth, glucose fermentation by many *Clostridium* species can result in production of a mixture of major fermentation products including acetate, butyrate, and ethanol [19]. Among these major metabolites, formation of acetate and butyrate accompanies with net production of hydrogen gas, whereas production of ethanol does not. Eqs. (1)–(3) summarize the stoichiometric relations between glucose and products formed during clostridial fermentation [19]:

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 4H_2 + 2CO_2, \quad (1)$$

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2H_2 + 2CO_2, \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2. \quad (3)$$

The International Water Association (IWA) task group for mathematical modeling of anaerobic digestion processes [24] developed the anaerobic digestion model no. 1 (ADM1) to describe the stoichiometry and kinetics of reactions occurred in anaerobic processes. In this study, the model structure of ADM1 was adopted and modified to apply in mathematical model simulations for clostridial glucose fermentation processes. For model simulations, glucose consumption by *Clostridium* species was calculated directly, and the corresponding changes in biomass, acetate, butyrate, ethanol, and hydrogen were calculated using the stoichiometric relationships shown in Table 1. For the rate of glucose consumption (r_{Glu}), a Monod-type kinetic expression, incorporating the empirical lower pH inhibition, was used and can be defined as $r_{Glu} = q_{Glu}^{max} S_{Glu} X/(K_{Glu} + S_{Glu}) \cdot I_{pH}$, where X denotes biomass concentration, S_{Glu} denotes residual glucose concentration, q_{Glu}^{max} denotes maximum specific glucose consumption rate, and K_{Glu} represents Monod half-saturation constant. The empirical lower pH inhibition term, I_{pH}, according to the ADM1, was defined as $I_{pH} = \exp(-3((pH - pH_{UL})/(pH_{UL} - pH_{LL}))^2)|_{pH<pH_{UL}}$, where pH_{UL} and pH_{LL} denote the upper limit at which the acidogenic bacteria are not inhibited ($I_{pH} = 1$ when $pH > pH_{UL}$), and the lower limit at which inhibition is complete, respectively. In the ADM1, the values of pH_{UL} and pH_{LL} are recommended to be 5.5 and 4, respectively, for acidogenic bacteria. Parameter estimation and model simulations were performed using the AQUASIM software package [25].

Table 1
Stoichiometric matrix of clostridial glucose fermentation

	S_{Glu}	S_{HAc}	S_{HBu}	S_{Eth}	S_{H_2}	X
Glucose consumption	-1	$(1-Y)f_{HAc}$	$(1-Y)f_{HBu}$	$(1-Y)f_{Eth}$	$(1-Y)f_{H2}$	Y

3. Results and discussion

3.1. BHP tests for C. acetobutylicum M121, C. butyricum ATCC19398, C. tyrobutyricum FYa102, and C. beijerinckii L9

The BHP test results for the pure cultures of *C. butyricum* ATCC19398 and *C. beijerinckii* L9 including pH, concentrations of glucose, biomass (in VSS), VFAs, and alcohols, and cumulative gas production are presented in Fig. 1. Obviously, both cultures fermented glucose, produced new biomass, and formed major fermentation products including acetate and butyrate. Production of hydrogen and carbon dioxide along with acetate and butyrate formation has been known as a typical metabolic path during fermentative hydrogen production by clostridial species [10,26,27]. Once the glucose was depleted, biomass growth, product formation, and gas production also ceased. Corresponding to VFA production, the medium pH decreased from 7.2 to a value between 4.6 and 5.0. Release of ammonia nitrogen was insignificant during the BHP test, indicating that peptone was primarily utilized for biosynthesis of biomass [28,29]. The patterns of BHP test results for *C. acetobutylicum* M121 and *C. tyrobutyricumi* FYa102 isolates were very similar to those observed for *C. butyricum* ATCC19398 and *C. beijerinckii* L9 as shown in Fig. 1, but with differences in quantities of fermentation end products. In addition to acetate and butyrate, *C. acetobutylicum* also produced a considerable amount of ethanol as previously reported [30].

The yields of fermentation end products in BHP tests are summarized in Table 2. The four *Clostridium* species examined in this study fermented glucose to produce biomass and to form common major products including acetate, butyrate, carbon dioxide, and hydrogen [19]. In addition to common products, *C. acetobutylicum* M121 produced ethanol with a yield of 0.19 mmol/mmol-glucose. Among four species tested, *C. tyrobutyricum* FYa102 and *C. beijerinckii* L9 possessed the highest and lowest biomass yield, respectively. In contrast, *C. beijerinckii* L9, had the highest hydrogen yield of 2.81 mmol/mmol-glucose, while *C. tyrobutyricum* FYa102 had the lowest hydrogen yield of 1.47 mmol/mmol-glucose.

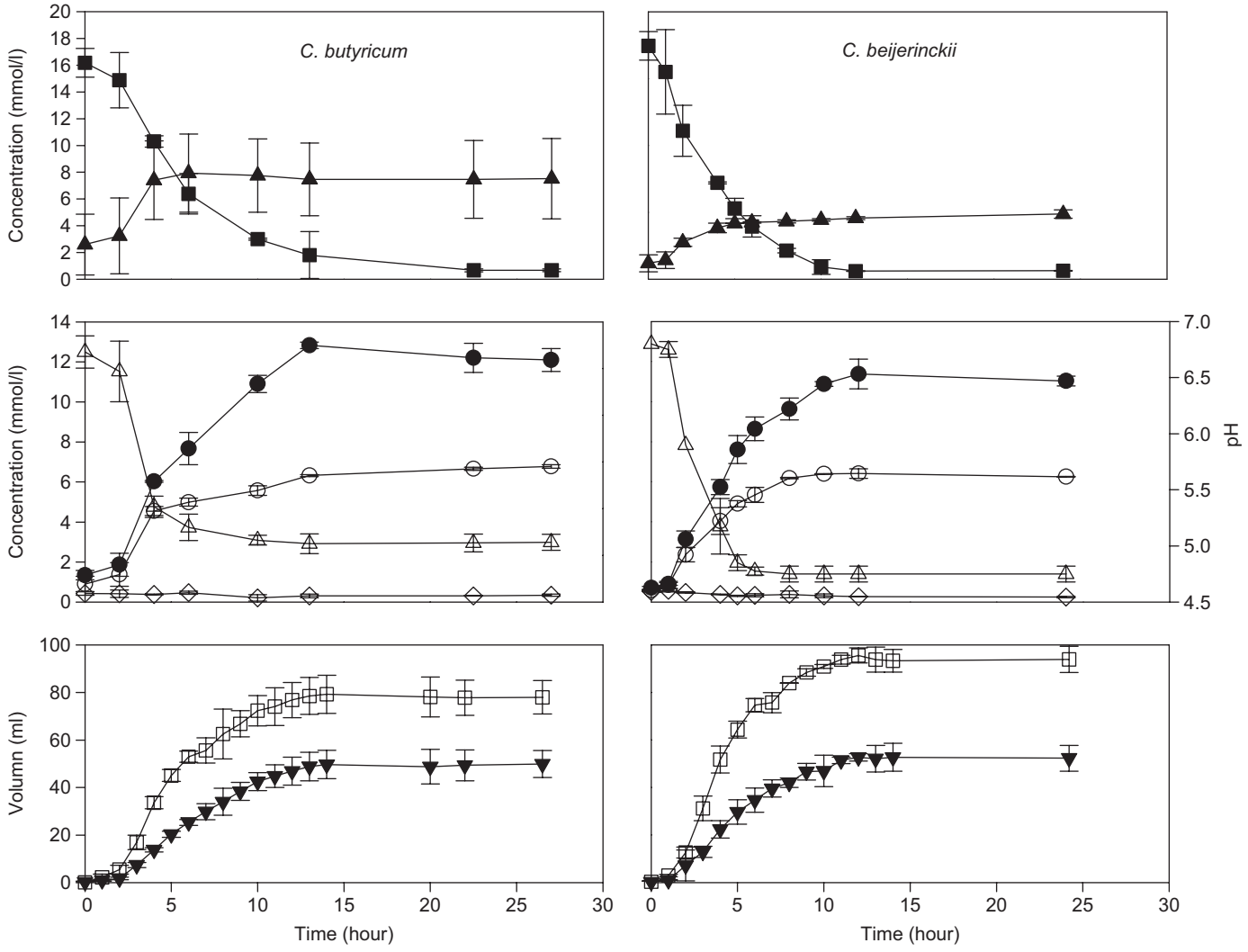

Fig. 1. The BHP test results for the *C. butyricum* ATCC19398 and *C. beijerinckii* L9. Glucose, ■; Biomass, ▲; Acetate, ○; Butyrate, ●; Ethanol, ◊; pH, △; Hydrogen, □; Carbon dioxide, ▼.

Table 2
Fermentation end product yields for *C. acetobutylicum*, *C. butyricum*, *C. tyrobutyricum*, and *C. beijerinckii* (values in parentheses represent standard errors obtained from duplicated experiments)

Product yield (mmol/mmol-glucose)	*C. acetobutylicum*	*C. butyricum*	*C. tyrobutyricum*	*C. beijerinckii*
$Y_{Biomass}$	0.2 (0.01)	0.34 (0.08)	0.46 (0.11)	0.23 (0.02)
Y_{H_2}	1.8 (0.02)	2.29 (0.21)	1.47 (0.73)	2.81 (0.04)
Y_{CO_2}	1.31 (0.01)	1.49 (0.19)	0.85 (0.69)	1.65 (0.04)
Y_{HLa}	0.02 (0.002)	0.05 (0.03)	0.01 (0.04)	0.01 (0.01)
Y_{HFo}	0.04 (0.002)	0.03 (0.03)	0.07 (0.05)	0.00
Y_{HAc}	0.43 (0.05)	0.36 (0.01)	0.42 (0.14)	0.34 (0.00)
Y_{HPr}	0.00	0.11 (0.01)	0.00	0.00
Y_{HBu}	0.67 (0.02)	0.67 (0.04)	0.37 (0.35)	0.62 (0.01)
$Y_{Ethanol}$	0.19 (0.11)	0.00	0.02 (0.01)	0.00
$Y_{Propanol}$	0.00	0.00	0.00	0.00
$Y_{Butanol}$	0.00	0.00	0.00	0.03 (0.01)
Y_{HBu}/Y_{HAc}	1.54	1.86	0.88	1.82
$Y_{H_2}/2(Y_{HAc}+Y_{HBu})$	0.84	1.11	0.93	1.46

The hydrogen yield for *C. butyricum* ATCC19398 was 2.29 mmol/mmol-glucose, which was in good agreement with the value of 2.4 mmol/mmol-hexose reported by Ueno et al. [5]. The theoretically maximum hydrogen yield from glucose fermentation can be estimated as 4 mol providing that acetic acid is the final product [1]. The reported hydrogen

Table 3
Estimated parameter values and their corresponded standard errors (in parentheses) for *C. acetobutylicum*, *C. butyricum*, *C. tyrobutyricum*, and *C. beijerinckii*

	C. acetobutylicum	*C. butyricum*	*C. tyrobutyricum*	*C. beijerinckii*
q_G^{max}	0.496	0.88	0.428	1.03
(mmol/mmol/h)	(0.043)	(0.096)	(0.031)	(0.06)
K_{Glu}	1	4.34	4	2.6
(mmol/l)	(0.011)	(1.8)	(0.4)	(0.2)
Y	0.311	0.46	0.498	0.229
(mmol/mmol)	(0.045)	(0.037)	(0.054)	(0.04)
f_{HAc}	0.65	0.65	0.714	0.464
(mmol/mmol)	(0.096)	(0.078)	(0.121)	(0.053)
f_{HBu}	0.87	1.219	0.744	0.83
(mmol/mmol)	(0.11)	(0.11)	(0.126)	(0.07)
f_{Eth}	0.341	0	0.032	0
(mmol/mmol)	(0.084)	(0.001)	(0.092)	(0.001)
f_{H_2}	2.69	4.17	2.79	3.66
(mmol/mmol)	(0.238)	(0.29)	(0.315)	(0.188)
f_{HBu}/f_{HAc}	1.34	1.88	1.04	1.79
$f_{H_2}/2(f_{HAc}+f_{HBu})$	0.88	1.12	0.96	1.41

production efficiencies from pure cultures varied from 22% to 57% (based on 4 mol of hydrogen per mole of glucose) with defined carbon substrates such as glucose [31]. In this work, the hydrogen production efficiency of *C. beijerinckii* L9 was about 70% ($2.81/4=0.7$), which was considerably higher than the values indicated in the literature.

The ratio of Y_{HBu} to Y_{HAc} (B/A ratio) was considered as an indicator for evaluating the efficiency of hydrogen production (Y_{H_2}) during glucose fermentation with mixed microflora [7,32]. Based on the stoichiometry of glucose fermentation as shown in Eqs. (1) and (2), more hydrogen is evolved from producing acetate than from butyrate production. However, results from our pure-culture studies and others [32] show that there seemed to be a strong positive correlation between the values of Y_{HBu}/Y_{HAc} ratio and the hydrogen yields for different *Clostridium* species (Table 2), although the fundamentals are not clear at this time. Jungermann et al. [10] introduced the ratio of $Y_{H_2}/2(Y_{HAc}+Y_{HBu})$ to determine the fermentation balance between acetate, butyrate, and hydrogen based on the stoichiometric correlation that 2 mol of hydrogen are formed per mole each of acetate and butyrate. In their study, the ratios obtained from glucose fermentation for *C. tyrobutyricum* FYa102 and *C. butyricum* L9 were constantly close to 1, confirming the stoichiometric relationships shown in Eqs. (1) and (2). Table 2 shows that the ratio of $Y_{H_2}/2(Y_{HAc}+Y_{HBu})$ for *C. beijerinckii* L9 was 1.46, which was substantially higher than those calculated for the other three *Clostridium* species (ranging from 0.84 to 1.11), indicating its high hydrogen production yield. In addition, this particularly high ratio may suggest the presence of different stoichiometries between acetate, butyrate, and hydrogen compared to those presented in Eqs. (1) and (2), while this still requires further validation.

3.2. Mathematical model simulation

The kinetic parameters and stoichiometric coefficients involved in the models for different *Clostridium* species were estimated by running the optimization routine against the experimental data sets using the numerical simulation software Aquasim [25]. Table 3 lists the estimated parameter values for *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9 and their corresponded standard errors (in parentheses). Moreover, the kinetic models with estimated parameters were used to generate predicted results as presented in Fig. 2. In general, the model was able to describe the trends of glucose consumption, biomass production, and fermentation product formation fairly well. Although the trend of product formation was considerably different for different *Clostridium* species, the kinetic model and estimated parameters provided satisfactory predictions of experimental observations shown in Fig. 2.

The estimated values of maximum specific glucose consumption rate (q_G^{max}) and Monod half-saturation constant (K_{Glu}) listed in Table 3 were comparable to those reported in ADM1 ($q_G^{max}=1.04$ mmol/mmol/h and $K_{Glu}=2.6$ mmol/l, respectively) [24]. The estimated kinetic parameters for different *Clostridium* species suggest that *C. beijerinckii* L9 possessed a kinetic advantage in competing glucose with the other three strains because of its highest q_G^{max} and relatively low K_{Glu} values. The estimated biomass yield coefficients (Y) were within the range of reported values for acid producing bacteria (0.239–0.68) [33–37], but were higher than that of ADM1 (0.083 mmol/mmol), probably due to the biomass yield contributed by peptone metabolism [29,38]. Using bioenergetic calculations according to McCarty [39] and assuming glucose as the sole substrate, a theoretical true yield of 0.278 mmol/mmol for acid producing bacteria was obtained and the value was comparable to the estimated values listed in Table 3. The yield coefficients of acetate, butyrate, and ethanol for the four *Clostridium* species varied from 0.464 to 0.714 mmol/mmol, 0.83 to 1.219 mmol/mmol, and 0 to 0.341 mmol/mmol, respectively, confirming the diverse distributions of fermentation end products for different *Clostridium* species [30].

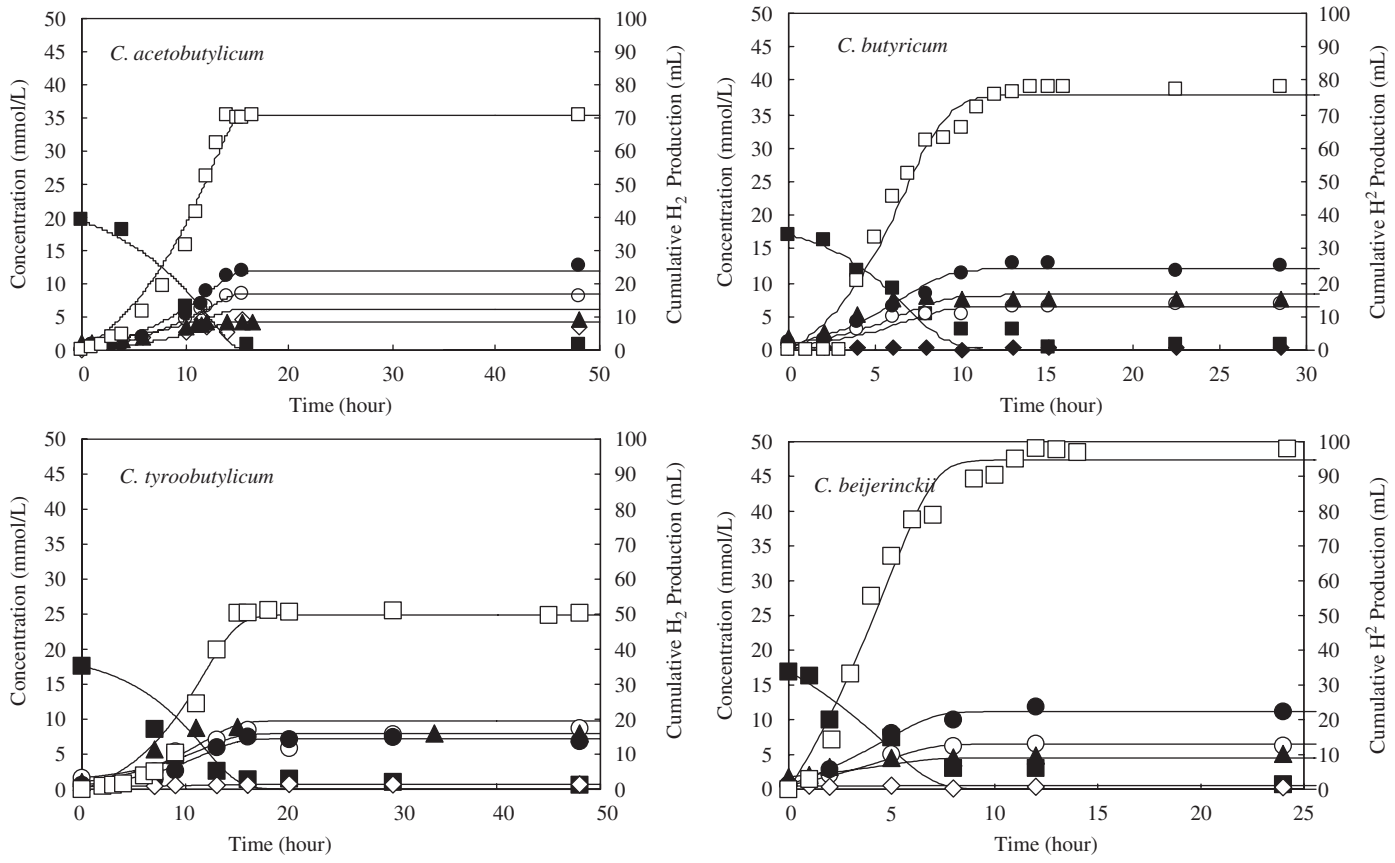

Fig. 2. Experimental (represented by symbols) and simulation (represented by solid lines) results of BHP tests for *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9. Glucose, ■; Biomass, ▲; Acetate, ◯; Butyrate, ●; Ethanol, ◊; Hydrogen, □.

Table 4
Correlation matrix for Y_{HBu}/Y_{HAc}, $Y_{H_2}/2(Y_{HAc}+Y_{HBu})$, f_{HBu}/f_{HAc}, $f_{H_2}/2(f_{HAc}+f_{HBu})$, and Y_{H_2}

	Y_{HBu}/Y_{HAc}	$Y_{H_2}/2(Y_{HAc}+Y_{HBu})$	f_{HBu}/f_{HAc}	$f_{H_2}/2(f_{HAc}+f_{HBu})$	Y_{H2}
Y_{HBu}/Y_{HAc}	1.000				
$Y_{H_2}/2(Y_{HAc}+Y_{HBu})$	0.571	1.000			
f_{HBu}/f_{HAc}	0.954	0.687	1.000		
$f_{H_2}/2(f_{HAc}+f_{HBu})$	0.547	1.000	0.669	1.000	
Y_{H2}	0.845	0.919	0.884	0.907	1.000

Similar to the concept of Y_{HBu}/Y_{HAc} and $Y_{H_2}/2(Y_{HAc}+Y_{HBu})$ ratios, the f_{HBu}/f_{HAc} and $f_{H_2}/2(f_{HAc}+f_{HBu})$ ratios calculated by estimated parameters are also presented in Table 3. To further validate the estimated parameters listed in Table 3, a statistical analysis on correlation between different calculated ratios was performed and the results are presented in Table 4. The correlation coefficients between Y_{HBu}/Y_{HAc} and f_{HBu}/f_{HAc} as well as $Y_{H_2}/2(Y_{HAc}+Y_{HBu})$ and $f_{H_2}/2(f_{HAc}+f_{HBu})$ were as high as 0.954 and 1.000, respectively, indicating the correctness of estimated parameters presented in Table 3. Furthermore, the robust correlation (0.845–0.919) between hydrogen production yield (Y_{H_2}) and different ratios suggests the potential application of these ratios as indicators for evaluating the efficiency of hydrogen production during glucose fermentation.

3.3. Glucose re-feeding experiments

The glucose re-feeding experiments were conducted to evaluate whether the termination of hydrogen production at the end of regular BHP tests for different *Clostridium* species was due to insufficient glucose or a metabolic shift. At the end of regular BHP tests, glucose and peptone were added into serum bottles to provide an additional 3 000 mg/l of glucose and 2000 mg/l of peptone as fermentation substrates. The results of glucose re-feeding experiment for *C. butyricum* ATCC19398 and *C. beijerinckii* L9 are presented in Fig. 3.

When glucose was added at the end of regular BHP test (25 h), the *C. butyricum* ATCC19398 started to consume glucose at 30 h until the end of experiment at 100 h. Corresponding to glucose consumption, substantial amounts of lactate and

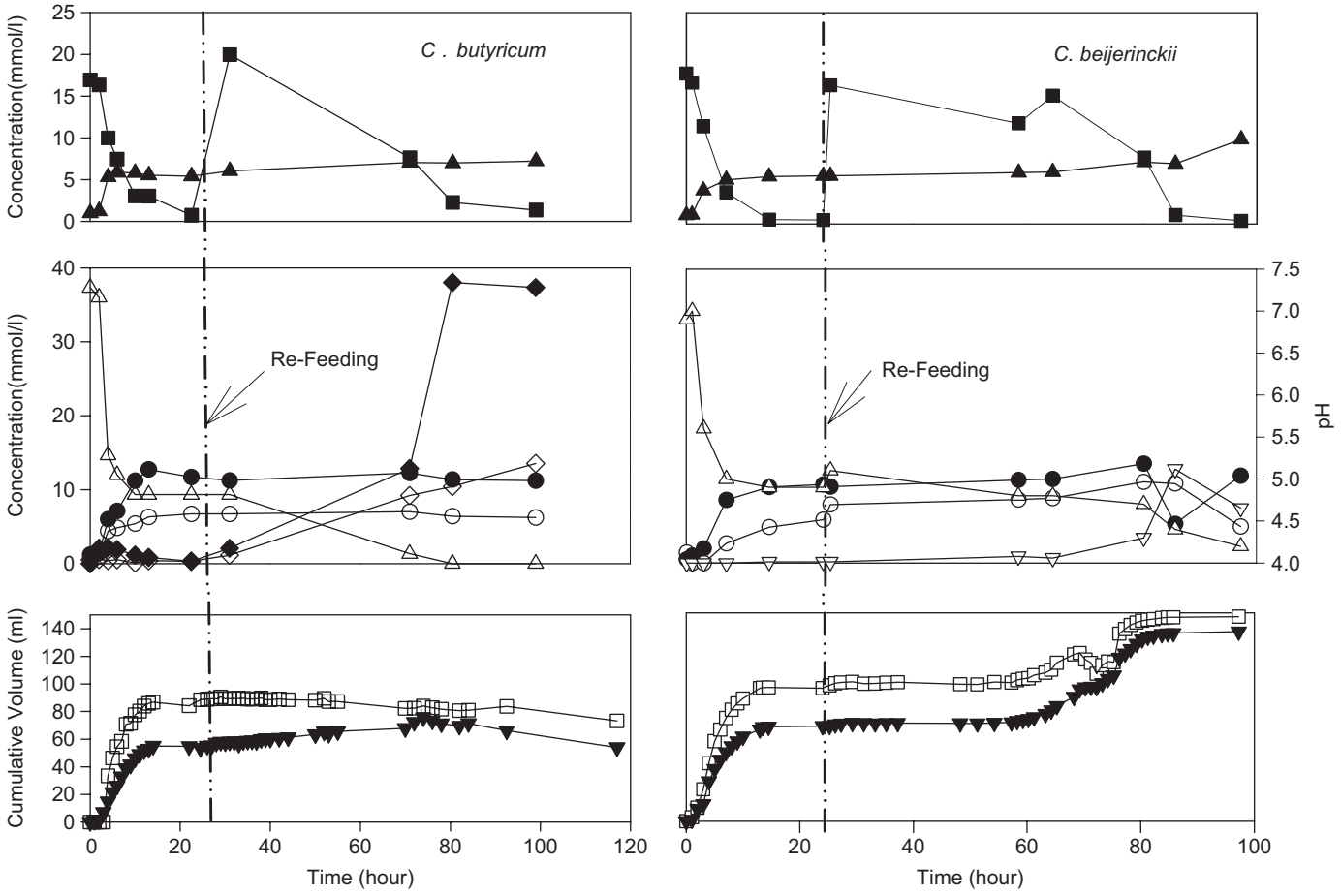

Fig. 3. The glucose re-feeding test results for the *C. butyricum* ATCC19398 and *C. beijerinckii* L9. Glucose, ■; Biomass, ▲; Acetate, ○; Butyrate, ●; Ethanol, ◊; pH, △; Hydrogen, □; Carbon dioxide, ▼; Lactate, ♦; Butanol, ▽.

ethanol were produced and the pH decreased from 4.6 to around 4 at 100 h. No significant production of acetate, butyrate, and hydrogen was observed after glucose re-feeding, indicating that the strain may undergo a metabolic shift from acetate/butyrate formation toward lactate/ethanol formation under stressed conditions with a low pH or a high acid end product concentration [1,20]. In contrast to the result observed for *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9 were able to ferment additional glucose during re-feeding tests to produce a significant amount of hydrogen, suggesting that insufficient glucose could be the reason for the termination of hydrogen production during regular BHP tests.

4. Conclusions

The BHP tests conducted for *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9 showed that clostridial glucose fermentation produced mainly butyrate (0.37–0.67 mmol/mmol glucose) and acetate (0.34–0.42 mmol/mmol glucose). A significant amount of hydrogen evolved accompanied with glucose degradation and acid production. Among the four cultures tested, *C. beijerinckii* L9 attained the highest hydrogen yield of 2.81 mmol/mmol-glucose. The kinetic model and estimated parameters applied in this study were able to accurately describe the degradation of glucose and production of biomass, butyrate, acetate, ethanol, and hydrogen observed in the batch tests, whereas the trend of product formation was considerably different for different *Clostridium* species. The estimated kinetic parameters for different *Clostridium* species suggest that *C. beijerinckii* L9 was kinetically favorable in competing glucose with the other three strains because of its highest q_G^{max} and relatively low K_{Glu} values. Results from glucose re-feeding experiments showed that *C. tyrobutyricum* FYa102 and *C. beijerinckii* L9 could utilize additional glucose to produce more hydrogen. The *C. butyricum* ATCC19398 was not able to produce hydrogen once the pH decreased to below 4.5 although additional glucose was available, presumably due to its metabolic shift from acetate/butyrate to lactate/ethanol production.

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