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Effect of Initial Glucose Concentrations on Carbon and Energy Balances in Hydrogen-Producing *Clostridium tyrobutyricum* JM1

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The carbon metabolism of newly isolated *Clostridium tyrobutyricum* JM1 was investigated at varying initial glucose concentrations (27.8–333.6 mM). Because an understanding of metabolic regulations was required to provide guidance for further effective metabolic design or optimization, in this case, maximizing hydrogen production, carbon and energy balances by *C. tyrobutyricum* JM1 were determined and applied in anaerobic glucose metabolism. The overall carbon distribution suggested that initial glucose concentrations had strong influence on the stoichiometric coefficients of products and the molar production of ATP on the formation of biomass. *C. tyrobutyricum* JM1 had a high capacity for hydrogen production at the initial glucose concentration of 222.4 mM with high concentrations of acetate and butyrate.

Keywords: *Clostridium tyrobutyricum*, carbon material balance, energy balance, carbon flow distribution, hydrogen production

Saccharolytic clostridia represent one of the largest genera of prokaryotes and satisfy the following four criteria: (a) able to form endospores; (b) must rely on the energy metabolism of obligate anaerobes; (c) unable to carry out a dissimilatory sulfate reduction; and (d) the cell wall must be Gram-positive [2]. *Clostridium tyrobutyricum*, one of the saccharolytic clostridia, is a low-G+C Gram-positive anaerobe and exhibits special metabolic routes to produce short-chain fatty acids and hydrogen gas from carbohydrates and amino acids [2, 13]. Hydrogen is recognized as a renewable and promising energy alternative for the future because it does not emit carbon-based gases and it has high specific energy content per unit mass [12]. Among biological hydrogen production processes, fermentative hydrogen production by clostridia proceeds from the anaerobic metabolism of pyruvate by the following electron transfer chains: pyruvate:ferredoxin oxidoreductase (PFOR), NADH: ferredoxin oxidoreductase (NFOR), and hydrogenase [23]. PFOR oxidizes pyruvate to acetyl-CoA and CO_2 with reduced ferredoxin, from which electrons are transferred to reduce protons for H_2 production [Eq. (1)]. It is known to function at the H_2 concentrations observed in a fermentative system [17, 23].

$$\text{Pyruvate}+\text{CoA}+2\text{Fd}_{ox} \xrightarrow{\text{PFOR}} \text{Acetyl-CoA}+\text{CO}_2+2\text{Fd}_{red} \quad (1)$$

NFOR recycles the NAD^+ by the re-oxidization of NADH and also produces reduced ferredoxin (Fd_{red}) related to H_2 evolution [17]. The Gibbs free energy for H_2 production from NADH varies with the following equation [15]:

$$\Delta G' = \Delta G^{0\prime} + RT' \ln \frac{[NAD^+](pH_2)}{[NADH]} \quad (2)$$

where G^0 is 18.14 kJ/mol [1, 32]. Finally, hydrogenase enzymes (H_2ase) catalyze the oxidation or evolution of molecular hydrogen from two protons (H^+) and electrons [28]:

$$H_2 \overset{\text{hydrogenase}}{\rightleftharpoons} 2H^+ + 2e^- \quad (3)$$

According to the protein sequence homology, the hydrogenase enzymes can be classified into four groups: [Ni-Fe]-hydrogenase (aerobic, anaerobic, and facultative anaerobic bacteria), [Ni-Fe-Se]-hydrogenase (sulfate-reducing bacteria), [FeFe]-hydrogenase (strict anaerobic bacteria), and metal-free hydrogenase (methanogens) [8]. In particular, [FeFe]-hydrogenase is the distinct class of hydrogenase responsible for hydrogen production; it has the unusual characteristic

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of which the H-cluster consists of a bimetallic center with two Fe atoms with CO and CN coordination [8, 28]. The coordination of the diatomic ligands results in low-spin Fe. Such a low oxidation state at the Fe center site facilitates the binding of hydrogen [31].

A major bottleneck in the fermentative processes is relatively lower hydrogen yields than those of a photosynthetic hydrogen production [27]. This is because fermentation processes have optimally evolved to produce not hydrogen, but cell biomass [17]. It was reported that the utilization of substrate as an energy source for bacterial growth as well as the oxidation of NADH (reducing equivalent) by the conversion of pyruvate to alcohol (ethanol or butanol) and reduced acids (lactate) were the main reasons for obtaining yields lower than the theoretical value [21, 27]. Microorganisms are known to change their metabolic pathway according to metabolites concentrations [27]. The metabolites are greatly influenced by many factors such as pH, temperature, and nutritional requirements [9, 26]. Most previous studies concentrated on the optimization of environmental conditions or the examination of the simple relationship between input and output for the improvement of hydrogen production. However, a prior understanding of metabolic regulation during the catabolism of glucose (one of the most common carbohydrates) is required to overcome the low yield of hydrogen. To date, information about which fractions of carbon are consumed for the synthesis of biomass and production of various by-products can be gained by only a few facultative anaerobes [5, 29]. Moreover, *C. tyrobutyricum* was primarily used for butyrate fermentation, not hydrogen production, and the information about carbon metabolism and quantitative analysis has not been reported.

The objective of this work is to investigate how an initial glucose concentration (S_o) influences an anaerobic metabolism through a carbon flow distribution determined by carbon material and energy balances in hydrogen-producing *C. tyrobutyricum* JM1 in batch cultivation. Glucose concentration plays an important role in the yield and production rate of hydrogen [14]. A low initial glucose concentration results in a low rate of the fermentation steps, and the fermentation time increases as the initial substrate concentration increases.

Materials and Methods

Culture and Media

Clostridium tyrobutyricum JM1 was isolated from a food waste treatment process [20]. Continuous operation of the cells in an immobilized bioreactor was an effective and stable approach for efficient utilization of carbon substrates with a good hydrogen-producing performance (maximum 7.2 l H_2/l·d and 223 ml H_2/g-hexose) [18]. The value was higher than those of other researches using various packed-bed bioreactor systems, in which biomass was either immobilized in granules or entrapped in packed media [18]. Therefore, the strain has high potential as a hydrogen-producing bacteria (HPB).

C. tyrobutyricum JM1 was precultured in a Reinforced Clostridial Medium (RCM) at 37°C for 24 h under anaerobic conditions. The RCM was composed of the following materials (all % w/v): meat extract, 1.0; peptone, 0.5; yeast extract, 0.3; D(+) glucose, 0.5; starch, 0.1; sodium chloride, 0.5; sodium acetate, 0.3; and L-cysteinium chloride, 0.05. The cells were harvested at the end of the exponential phase, washed with a PBS (phosphate-buffered saline) buffer, and used as inocula for the batch experiments.

Operation of the Anaerobic Reactor for the Batch Experiment

To investigate the effects of initial glucose concentrations on the carbon metabolic pathways, batch fermentation was aseptically performed in an anaerobic reactor with a total volume of 5 l. The reactor was filled with CGM (Clostridial Growth Medium) up to the working volume, 3 l. The medium, supplemented with variable glucose concentrations (27.8–333.6 mM), was composed of the following materials (all % w/v): peptone, 0.4; sodium chloride, 0.3; potassium monophosphate, 0.1; potassium diphosphate 0.1; magnesium chloride, 0.03; ferrous sulfate heptahydrate, 0.03; and L-cysteinium chloride, 0.05. The 5 ml of concentrated cells previously selected from batch systems was inoculated and incubated under optimal conditions (pH=6.3 and T=37°C) [2, 19]. Oxygen within the headspace of the reactor was removed by flushing it with nitrogen gas for 10 min. The anaerobic reactor was agitated at 150 rpm and managed at 37°C. The pH of the contents was maintained in the range of 6.3±0.1 by automatic titration with 5 N NaOH and 5 N HCl solutions.

Determination of Carbon Material and Energy Balances

The experimental data of the final metabolites' concentrations (acetate, butyrate, lactate, ethanol, carbon dioxide, and hydrogen) and biomass were utilized to determine the carbon material and energy balances. The glucose conversion stoichiometric equation is presented in the Appendix. The balance equations were based on the consumption of 1 mol glucose and determined by analyses of all the measurable metabolites, substrate, and cell mass during glucose fermentation. As shown in the scheme of glucose catabolism, it was assumed that (i) two molecules of ATP were generated from glucose to pyruvate *via* an Embden-Meyerhof-Parnas (EMP) pathway, (ii) two extra ATP molecules per glucose equivalent were formed by AK (acetate kinase), (iii) two NAD^+ molecules per glucose eq. were reduced to $NADH_2^+$ by GAPDH (glyceraldehyde-3-phosphate dehydrogenase, not shown in Fig. 1) during the EMP pathway [11], (iv) two NAD^+ per glucose eq. were produced by BHBD (β-hydroxylbutyryl-CoA dehydrogenase) and BCD (butyryl-CoA dehydrogenase), (v) two extra NAD^+ were formed by LDH (lactate dehydrogenase) (vi) four NAD^+ molecules were generated by ALDH (acetaldehyde dehydrogenase) and ADH (alcohol dehydrogenase), and (vii) the carbon fraction for microbial growth was estimated assuming the dry cell composition reported by Zeng *et al.* [35] (Fig. 1).

In the generalized degree of reduction balance, the coefficient multiplying the yield is called the degree of reduction of the corresponding compound. The degree of reduction is 4.2 for biomass, 6 for ethanol, 4 for glucose, and 0 for water, ammonia, and carbon dioxide [33]. With the generalization of Roels, the degree of reduction of the nitrogen-containing compounds depends on the nitrogen source used [33]. In most cases, ammonium is used as

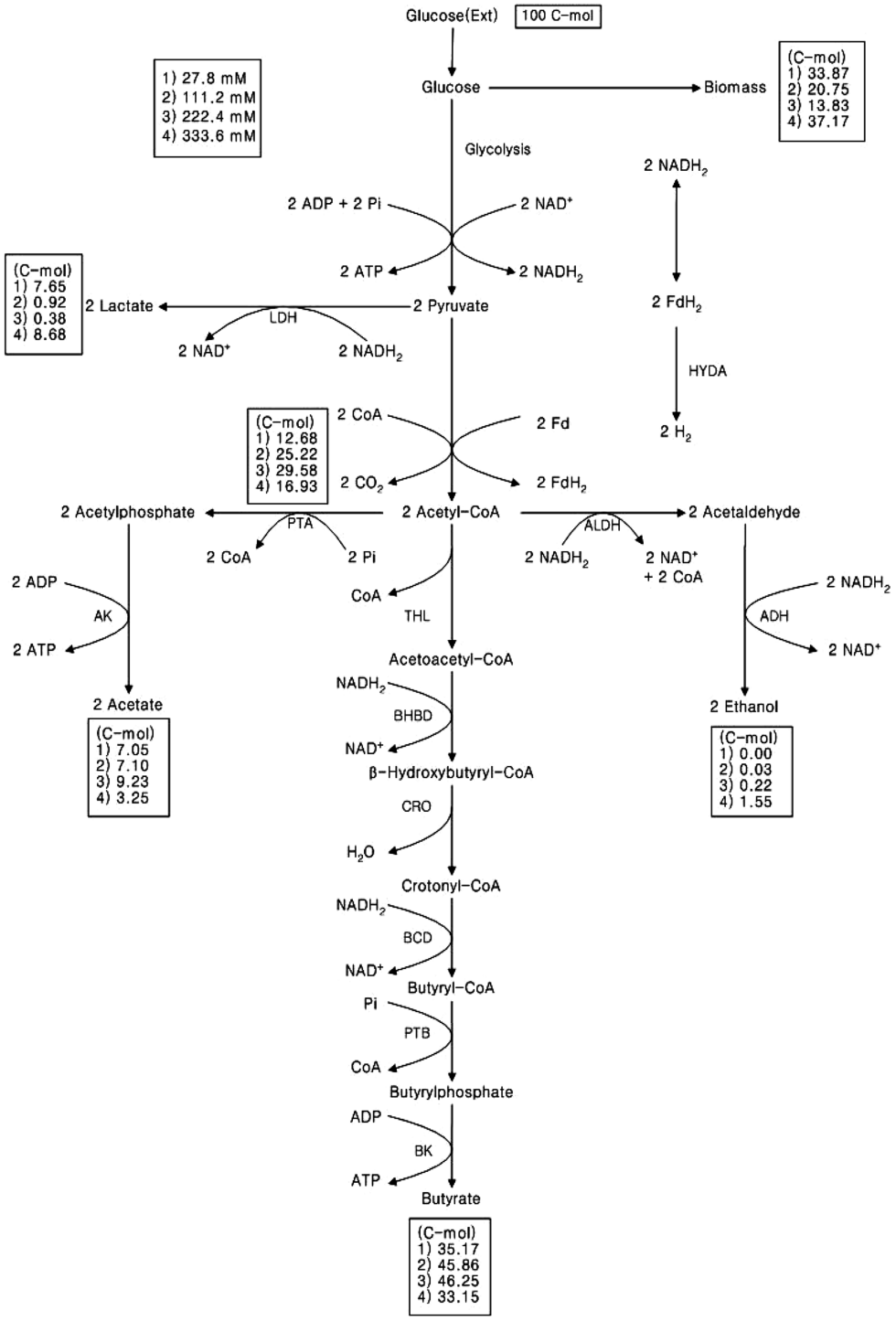

Fig. 1. The primary metabolic network map of *C. tyrobutyricum* JM1 showing experimental end products yields under varying initial glucose concentrations (27.8, 111.2, 222.4, and 333.6 mM).
All values are given in C-mol carbon transferred and refer to the consumption of 100 C-mol glucose. Enzymes: HYDA, hydrogenase; PTA, phosphotransacetylase; AK, acetate kinase; THL, thiolase; BHBD, β-hydroxybutyryl-CoA dehydrogenase; CRO, crotonase; BCD, butyryl-CoA dehydrogenase; PTB, phosphotransbutyrylase; BK, butyrate kinase; LDH, L-lactate dehydrogenase; ALDH, acetaldehyde dehydrogenase; and ADH, alcohol dehydrogenase.

either the sole nitrogen source or in combination with another nitrogen source, yielding the following general expression for the degree of reduction k of a compound with the elemental composition $CH_aO_bN_c$: k=4+a−2b−3c [33].

Analytic Methods

The biogas and liquid suspension from the anaerobic reactor were constantly sampled for analyses. The total volume of evolved gases was measured using a water displacement method. The biogas composition was analyzed using gas chromatography (model 6890N; Agilent Inc., U.S.A.), as previously described [19]. The biogas consisted of H_2 and CO_2. Collected effluents were centrifuged at 8,000 ×*g* for 10 min, and then the supernatants were acidified by formate followed by filtration through a membrane of 0.2-μm pore size before analysis. The concentrations of ethanol and volatile fatty acids (VFAs) excluding lactate were analyzed using gas chromatography equipped with a flame ionization detector (FID) and a HP-INNOWax capillary column, with helium as a carrier gas at 2.2 ml/min. Both the injector and detector were kept at 250°C, while the oven was held at 60°C for 1 min, heated to 220°C at the heating rate of 10°C/min, and maintained at 220°C. Glucose, pyruvate, and lactate were analyzed using high-performance liquid chromatography (Model 1100; Agilent Inc.) with a refractive index detector (RID) and an Aminex HPX-87H column (300 mm×7.8 mm), and using 8 mN H_2SO_4 as the mobile phase. Cell growth was analyzed spectrophotometrically at 600 nm and calibrated against a dry cell weight measurement (1 OD_{600}=0.83 g-DW/l). For the analysis of the dry cell weight, 20-ml samples were centrifuged for 5 min at 8,000 ×*g*, washed with a PBS buffer, and dried at 105°C to a constant weight. The carbon fraction consumed for growth can be estimated by assuming the elemental composition of the biomass ($CH_{1.75}O_{0.5}N_{0.25}$) and neglecting the amount of CO_2 produced in biomass synthesis [5, 35].

Results and Discussion

Construction of the Metabolic Pathway of *C. tyrobutyricum* JM1

The primary metabolic network map of *C. tyrobutyricum* JM1 is shown in Fig. 1. It involves the glycolysis of glucose to pyruvate *via* the EMP pathway. Pyruvate is then oxidized to acetyl-CoA, which is a branch-point intermediate located at the node dividing three pathways for the production of acetate, butyrate, and ethanol [23]. Acetate is produced in two steps, and is catalyzed by phosphotransferase (PTA) and acetate kinase (AK) from acetyl-CoA. Acetyl-CoA can also be converted to butyryl-CoA, which is the precursor of butyrate [10]. Butyrate is also produced by the enzymes of phosphotransbutyrylase (PTB) and butyrate kinase (BK) from butyryl-CoA. ATP, required for biomass synthesis, is generated by the steps catalyzed by AK and BK. Depending on environmental conditions, pyruvate could be mainly converted to lactate by lactate dehydrogenase (LDH) or acetyl-CoA, with the cogeneration of acetate, butyrate, and ethanol [38]. *C. tyrobutyricum* is known not to produce solvents, such as acetone and butanol [2]. Therefore, the microorganism does not have the metabolic shift from acidogenesis to solventogenesis. Aside from the solvents, various volatile fatty acids such as caproate and valerate were excluded from the metabolic pathway of *C. tyrobutyricum* JM1 owing to their low concentrations.

Acetate and butyrate are formed during H_2-producing fermentation as per the following reactions:

$$\text{Glucose}+2H_2O\rightarrow 2\text{Acetate}+2CO_2+4H_2 \quad \Delta G^{0\prime}=-182.4\ \text{kJ} \tag{4}$$

$$\text{Glucose}\rightarrow\text{Butyrate}+2CO_2+2H_2\ \Delta G^{0\prime}=-257.1\ \text{kJ} \tag{5}$$

Approximately 50% of all clostridia isolated to date are known to carry out butyrate fermentation, just as does *C. tyrobutyricum* [17]. In the metabolic pathway of *C. tyrobutyricum*, some of the electrons from ferredoxin were transferred to H^+ for H_2 production on this first pathway. Others were transferred to NAD^+ for $NADH_2^+$ generation. All $NADH_2^+$-containing reducing equivalents produced under the glycolytic pathway would assumably produce H_2 by hydrogenase on the second pathway [4]. Furthermore, the small amount of CO_2 produced from general decarboxylation enzymes involved in biomass synthesis were ignored, and the pathways to acetate, butyrate, ethanol, and lactate generated ATP for synthesis and maintenance of the biomass [16].

Carbon Material and Energy Balances at Varying Initial Glucose Concentrations

pH is one of the most important factors in hydrogen production owing to its effects on FeFe-hydrogenase activity, metabolic pathways, and the duration of lag phase [7, 24, 25]. It was reported that low initial pH values of 4.0–4.5 cause longer lag periods [22]. On the other hand, high initial pH values such as 9.0 decrease lag time, but have a lower yield of hydrogen production [37]. It is known that the effect of pH is due to the change of the ionization state of the components in enzymatic reactions [14]. Temperature also affects the maximum specific growth, substrate utilization rate, and the metabolic pathway of the microorganisms, resulting in a shift of by-product compositions [24, 34]. Therefore, both the pH and temperature in all experiments were controlled at 6.3 and 37°C, respectively [2, 19].

All values are given in C-mol carbon transferred and refer to the consumption of 100 C-mol glucose as illustrated in Fig. 1. The predominant fermentation end products of *C. tyrobutyricum* glucose catabolism were butyrate, acetate, and lactate, whereas the production of ethanol was relatively low. When the percentages of acetate and butyrate with concomitant release of H_2 and CO_2 were high, those of the biomass and lactate were low (Tables 1 and 2). It was reported that butyrate is an inhibitor to microbial growth, and the accumulation of acetate (>15–22 mM) also inhibits biomass synthesis [3, 38]. Pyruvate can be converted to lactate by lactate dehydrogenase (LDH) or acetyl-CoA, with the cogeneration of acetate and butyrate. Therefore, the shifts of metabolisms to butyrate or acetate led to a low concentration of lactate. It was demonstrated that products

Table 1. Carbon material balance[a] of batch anaerobic fermentation of 1 mol glucose by *C. tyrobutyricum* JM1, carried out at varying initial glucose concentrations (values are given in C-mol).

S_o (mM)	27.8	111.2	222.4	333.6
Reactant				
Glucose	6.00	6.00	6.00	6.00
Products				
Acetate	0.423	0.426	0.554	0.195
Butyrate	2.110	2.752	2.775	1.989
Lactate	0.459	0.055	0.023	0.521
Ethanol	0.000	0.002	0.013	0.093
Biomass	2.032	1.245	0.830	2.230
CO_2 [b]	0.761	1.513	1.775	1.016
Total products	5.785	5.993	5.970	6.044
Error (%)	−3.5	−0.1	−0.5	0.7

[a]Values are the averages of three different experiments.
[b]CO_2 in the liquid phase was ignored.

such as butyrate and acetate corresponded to an accumulation of intracellular $NADH_2^+$, leading to a high $NADH_2^+/NAD^+$ ratio [30]. On the other hand, lactate is a reduced form produced by the consumption of $NADH_2^+$ to NAD^+. The low concentration of lactate would be caused by an increased flux of carbon towards the pathways for butyrate and acetate (Fig. 1). These pathways also induced the increased formation of reducing equivalents ($NADH_2^+$), which could also regulate the increased production of hydrogen by NFOR and hydrogenase. In addition, extracellular pyruvate was not detected in all experiments, suggesting that the flow through the PFOR was not limited [16]. The validity of this approach was confirmed by the check of carbon material and energy balances, as shown in Tables 1 and 2, for which one can estimate relative errors less than 3.5% and 3.2%, respectively. The fractional carbon distributions in various metabolites were made up of

Table 2. Energy balance of batch anaerobic fermentation of 1 mol glucose by *C. tyrobutyricum* JM1, carried out at varying initial glucose concentrations (standard deviation was within 5%).

S_o (mM)	27.8	111.2	222.4	333.6
Reactants				
Glucose	24.00	24.00	24.00	24.00
Products				
Acetate	1.694	1.704	2.214	0.780
Butyrate	10.550	13.760	13.876	9.947
Lactate	1.839	0.219	0.095	2.085
Ethanol	0.000	0.013	0.078	0.560
Biomass	8.128	4.981	3.321	8.923
Hydrogen	2.365	3.241	3.641	1.730
Total products	24.576	23.918	23.225	24.025
Error (%)	2.4	−0.3	−3.2	0.1

Table 3. Stoichiometric coefficients and products molar yields (mol/mol glucose) at varying initial glucose concentrations.

S_o (mM)	27.8	111.2	222.4	333.6
Coefficient				
α	0.076	0.009	0.004	0.086
β	0.632	0.794	0.835	0.568
β_1	0.105	0.106	0.138	0.048
β_2	0.527	0.688	0.694	0.497
β_3	0.000	0.0005	0.003	0.023
γ	0.084	0.051	0.034	0.092
Products yields				
$Y_{X/G}$	0.507	0.310	0.207	0.556
$Y_{X/ATP}$	0.234	0.123	0.078	0.291

See Appendix for calculating stoichiometric coefficients and molar yields.

the following ranges depending on the initial glucose concentrations, as shown in Fig. 1: acetate (3.25–9.23%), butyrate (33.15–46.25%), lactate (0.38–8.68%), ethanol (0.00–1.55%), biomass (13.83–37.17%), and CO_2 (12.68–29.58%). The majority of fractions of the metabolites produced were made up of butyrate, which could explain why *C. tyrobutyricum* is a good producer of butyrate. The pathway is similar to that of *C. butyricum*, which is a classic acid producer and ferments carbohydrates into butyrate, acetate, CO_2, and hydrogen [4]. In the case of a metabolite such as ethanol, the carbon distribution was below 1.55% regardless of the initial glucose concentrations. As the glucose concentration increased from 27.8 to 222.4 mM, butyrate formation increased from 2.110 to 2.775 C-mol, but rapidly decreased to 1.989 C-mol beyond this threshold. This occurrence could likely be due to a product inhibition phenomenon. It is known that higher butyrate concentrations induce lower yields and productivities of butyrate owing to butyrate inhibition [38]. The Appendix and Table 3 show the detailed metabolic reactions and their stoichiometric coefficients, respectively. The ultimate molar yields of products per mole of consumed glucose are presented in Table 3. The initial glucose concentration had strong effects on the formation of the fermentation products (butyrate, acetate, lactate, ethanol, carbon dioxide, hydrogen, and biomass). From S_o concentrations of 27.8 to 222.4 mM, acetate (β_1) and butyrate (β_2) production increased from 0.105 to 0.138 $mol_{acetate}/mol_G$ and from 0.527 to 0.694 $mol_{butyrate}/mol_G$, respectively, whereas the growth (γ) decreased from 0.084 to 0.034 C-mol_{DW}/mol_G. Therefore, hydrogen production increased by the increases of butyrate and acetate concentrations and decrease of cell mass at S_o concentration of 222.4 mM. Lactate (α) was also sensitive to S_o variations, decreasing from 0.076 (27.8 mM) to 0.004 $mol_{lactate}/mol_G$ (222.4 mM), but increasing beyond this threshold. High concentration of lactate by the activation of lactate dehydrogenase and consumption of $NADH_2^+$ can inhibit the carbon flow to acetate and butyrate *via* acetyl-

CoA, and further hydrogen production. Therefore, reduction of the carbon flux to lactate through deleting lactate dehydrogenase activity can improve hydrogen production. Molar production of ethanol (β_3) increased to 0.023 $mol_{ethanol}/mol_G$ with increasing S_o to 333.6 mM.

When butyrate and acetate are the main metabolites, ATP is generated in fermentative processes. In Table 3, the value of $Y_{X/ATP}$ under anaerobic conditions (average 0.18 C-mol_{DW}/mol_{ATP}) was lower than under aerobic conditions by *Aerobacter aerogenes* (0.35–1.0 C-mol_{DW}/mol_{ATP}), confirming that the synthesis of biomass under anaerobic conditions consumes more ATP than in the presence of oxygen [5, 6, 36]. The values of $Y_{ATP/G}$ and $Y_{X/G}$ have been used to estimate the average consumption of ATP for total synthesis of biomass precursors and their subsequent polymerization to biomass [5]. As shown in Table 3, the value of $Y_{X/ATP}$ (C-mol_{DW}/mol_{ATP}) at S_o concentration of 222.4 mM was lower than those of the remaining glucose concentrations, which indicated more energy should be required to shift the metabolism from metabolite production related to hydrogen to biomass synthesis. Therefore, the S_o concentration of 222.4 mM was advantageous in the production of hydrogen, along with concomitant formation of acetate and butyrate.

Carbon flow distributions were carried out to determine the shifts of carbon source according to the initial glucose concentrations. This is the first approach to investigate the regulation of the glucose metabolism of hydrogen-producing *Clostridium tyrobutyricum*. The validity of the data and the primary metabolic network model were confirmed from carbon material and energy balances. The analyses revealed that the initial glucose concentration of 222.4 mM was advantageous in the production of hydrogen, along with concomitant formation of acetate and butyrate. The condition activated a carbon metabolism yielding low biomass with low energetic growth efficiencies. Finally, reduction of the carbon flow to lactate through blocking the lactate dehydrogenase (LDH) activity can be expected to improve hydrogen production.

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APPENDIX: *CLOSTRIDIUM TYROBUTYRICUM* JM1 METABOLISM

Lactate Production

$$\alpha Glucose + 2\alpha NAD^+ + 2\alpha ADP + 2\alpha Pi \rightarrow 2\alpha Pyruvate + 2\alpha NADH_2^+ + 2\alpha ATP + 2\alpha H_2O \quad (1)$$

$$2\alpha Pyruvate + 2\alpha NADH_2^+ \rightarrow 2\alpha Lactate + 2\alpha NAD^+ \quad (2)$$

Net [Eqs. (1) and (2)]:

$$\alpha Glucose + 2\alpha ADP + 2\alpha Pi \rightarrow 2\alpha Lactate + 2\alpha ATP + 2\alpha H_2O \quad (3)$$

Pyruvate-Ferredoxin Oxidoreductase ($\beta=\beta_1+\beta_2+\beta_3$)

$$\beta Glucose + 2\beta NAD^+ + 2\beta ADP + 2\beta P_i \rightarrow 2\beta Pyruvate + 2\beta NADH_2^+ + 2\beta ATP + 2\beta H_2O \quad (4)$$

$$2\beta Pyruvate + 2\beta CoA + 2\beta Fd \rightarrow 2\beta Acetyl\text{-}CoA + 2\beta CO_2 + 2\beta FdH_2 \quad (5)$$

Net [Eqs. (4) and (5)]:

$$\beta Glucose + 2\beta NAD^+ + 2\beta ADP + 2\beta P_i + 2\beta CoA + 2\beta Fd \rightarrow 2\beta FdH_2 + 2\beta NADH_2^+ + 2\beta ATP + 2\beta H_2O + 2\beta CO_2 + 2\beta Acetyl\text{-}CoA \quad (6)$$

Hydrogen Production (Ferredoxin: Hydrogenase Activity)

$$2\beta FdH_2 \rightarrow 2\beta Fd + 2\beta H_2 \quad (7)$$

Acetate Production

$$2\beta_1 Acetyl\text{-}CoA + 2\beta_1 Pi \rightarrow 2\beta_1 Acetylphosphate + 2\beta_1 CoA \quad (8)$$

$$2\beta_1 Acetylphosphate + 2\beta_1 ADP \rightarrow 2\beta_1 Acetate + 2\beta_1 ATP \quad (9)$$

Net [Eqs. (8) and (9)]:

$$2\beta_1 Acetyl\text{-}CoA + 2\beta_1 Pi + 2\beta_1 ADP \rightarrow 2\beta_1 Acetate + 2\beta_1 CoA + 2\beta_1 ATP \quad (10)$$

Butyrate Production

$$2\beta_2 Acetyl\text{-}CoA \rightarrow \beta_2 Acetoacetyl\text{-}CoA + \beta_2 CoA \quad (11)$$

$$\beta_2 Acetoacetyl\text{-}CoA + \beta_2 \rightarrow NADH_2^+ \rightarrow \beta_2 3\text{-}Hydroxybutyryl\text{-}CoA + \beta_2 NAD^+ \quad (12)$$

$$\beta_2 3\text{-}Hydroxybutyryl\text{-}CoA \rightarrow \beta_2 Crotonyl\text{-}CoA + \beta_2 H_2O \quad (13)$$

$$\beta_2 Crotonyl\text{-}CoA + \beta_2\ NADH_2^+ \rightarrow \beta_2 Butyryl\text{-}CoA + \beta_2 NAD^+ \quad (14)$$

$$\beta_2 Butyryl\text{-}CoA + \beta_2 Pi \rightarrow \beta_2 Butyrylphosphate + \beta_2 CoA \quad (15)$$

$$\beta_2 Butyrylphosphate + \beta_2 ADP \rightarrow \beta_2 Butyrate + \beta_2 ATP \quad (16)$$

Net [Eqs. (11)–(16)]:

$$2\beta_2 Acetyl\text{-}CoA + 2\beta_2 NADH_2^+ + \beta_2 Pi + \beta_2 ADP \rightarrow \beta_2 Butyrate + 2\beta_2 CoA + 2\beta_2 NAD^+ + \beta_2 ATP + \beta_2 H_2O \quad (17)$$

Ethanol Production

$$2\beta_3 Acetyl\text{-}CoA + 2\beta_3 NADH_2^+ \rightarrow 2\beta_3 Acetaldehyde + 2\beta_3 NAD^+ + 2\beta_3 CoA \quad (18)$$

$$2\beta_3 Acetaldehyde + 2\beta_3 NADH_2^+ \rightarrow 2\beta_3 Ethanol + 2\beta_3 NAD^+ \quad (19)$$

Net [Eqs. (18) and (19)]:

$$2\beta_3\text{Acetyl-CoA}+4\beta_3\text{NADH}_2^+ \rightarrow 2\beta_3\text{Ethanol}+4\beta_3\text{NAD}^++2\beta_3\text{CoA} \quad (20)$$

Net [Eqs. (6), (7), (10), (17) and (20)]:

$$\beta\text{Glucose}+(2\beta-2\beta_2-4\beta_3)\ \text{NAD}^+ +(2\beta+2\beta_1+\beta_2)\ \text{ADP}+(2\beta+2\beta_1+\beta_2)\ \text{P}_i \rightarrow 2\beta_1\text{Acetate}+\beta_2\text{Butyrate}+2\beta_3\text{Ethanol}+2\beta\text{CO}_2 +2\beta\text{H}_2+(2\beta-2\beta_2-4\beta_3)\ \text{NADH}_2^++(2\beta+2\beta_1+\beta_2)\ \text{ATP} +(2\beta+\beta_2)\ \text{H}_2\text{O} \quad (21)$$

Cell Growth

$$\gamma\text{Glucose}+1.5\gamma\text{NH}_3+0.873\gamma\ \text{NADH}_2^+ \rightarrow 6\gamma\text{CH}_{1.75}\text{O}_{0.5}\text{N}_{0.25}+3\gamma\text{H}_2\text{O}+0.873\gamma\ \text{NAD}^+ \quad (22)$$

Formation of H_2 Through $NADH_2$: The reducing equivalents generated on all the metabolic pathways are used to produce molecular hydrogen by hydrogenase [4]

$$(2\beta-2\beta_2-4\beta_3-0.873\gamma)\ \text{NADH}_2^+ \rightarrow(2\beta-2\beta_2-4\beta_3-0.873\gamma)\ \text{NAD}^+ +(2\beta-2\beta_2-4\beta_3-0.873\gamma)\ \text{H}_2 \quad (23)$$

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Net [Eqs. (3), (21), (22) and (23)], where $\alpha+\beta+\gamma=1$ and $\beta=\beta_1+\beta_2+\beta_3$:

$$\text{Glucose}+(2\alpha+2\beta+2\beta_1+\beta_2)\ \text{ADP}+(2\alpha+2\beta+2\beta_1+\beta_2)\ \text{Pi} +1.5\gamma\text{NH}_3\rightarrow 2\alpha\text{Lactate}+2\beta_1\text{Acetate}+\beta_2\text{Butyrate} +2\beta_3\text{Ethanol}+2\beta\text{CO}_2+(4\beta-2\beta_2-4\beta_3-0.873\gamma)\ \text{H}_2 +(2\alpha+2\beta+2\beta_1+\beta_2)\ \text{ATP}+(2\alpha+2\beta+\beta_2+3\gamma)\ \text{H}_2\text{O} +6\gamma\text{CH}_{1.75}\text{O}_{0.5}\text{N}_{0.25} \quad (24)$$

NOMENCLATURE

S_o : initial glucose concentration, mM
X : biomass concentration, g_{DW}/l
$Y_{ATP/G}$: yield of ATP production on glucose consumption, mol_{ATP}/mol_G
$Y_{X/G}$: yield of growth on glucose consumption, C-mol_{DW}/mol_G
$Y_{X/ATP}$: yield of growth on ATP production, C-mol_{DW}/mol_{ATP}
k : degree of reduction

Greek Symbols

α : molar production of lactate, $mol_{lactate}/mol_G$ [Eq. (3)]
β : molar production of pyruvate, $mol_{pyruvate}/mol_G$ [Eq. (4)]
β_1 : molar production of acetate, $mol_{acetate}/mol_G$ [Eq. (10)]
β_2 : molar production of butyrate, $mol_{butyrate}/mol_G$ [Eq. (17)]
β_3 : molar production of ethanol, $mol_{ethanol}/mol_G$ [Eq. (20)]
γ : molar production of biomass, C-mol_{DW}/mol_G [Eq. (22)]

Subscripts

ATP : adenosine triphosphate
ADP : adenosine diphosphate
DW : dry weight
G : glucose

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