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A Modified Metabolic Model for Mixed Culture Fermentation With Energy Conserving Electron Bifurcation Reaction and Metabolite Transport Energy

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ABSTRACT: A modified metabolic model for mixed culture fermentation (MCF) is proposed with the consideration of an energy conserving electron bifurcation reaction and the transport energy of metabolites. The production of H_2 related to NADH/NAD^+ and Fdred/Fdox is proposed to be divided in three processes in view of energy conserving electron bifurcation reaction. This assumption could fine-tune the intracellular redox balance and regulate the distribution of metabolites. With respect to metabolite transport energy, the proton motive force is considered to be constant, while the transport rate coefficient is proposed to be proportional to the octanol–water partition coefficient. The modeling results for a glucose fermentation in a continuous stirred tank reactor show that the metabolite distribution is consistent with the literature: (1) acetate, butyrate, and ethanol are main products at acidic pH, while the production shifts to acetate and propionate at neutral and alkali pH; (2) the main products acetate, ethanol, and butyrate shift to ethanol at higher glucose concentration; (3) the changes for acetate and butyrate are following an increasing hydrogen partial pressure. The findings demonstrate that our modified model is more realistic than previous proposed model concepts. It also indicates that inclusion of an energy conserving electron bifurcation reaction and metabolite transport energy for MCF is sound in the viewpoint of biochemistry and physiology.

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KEYWORDS: metabolic modeling; variable stoichiometry; mixed culture fermentation; energy conserving electron bifurcation reaction; metabolite transport energy; transport rate coefficient

Introduction

Due to a worldwide shortage of suitable substrate and competition with food, anaerobic mixed culture fermentation (MCF) of waste materials has been recognized as a promising approach to realize resources recovery and valuable chemicals production (Kleerebezem and van Loosdrecht, 2007; Weusthuis et al., 2011). To better develop and operate such open fermentations it is desirable to construct a metabolic model for anaerobic MCF (Rodriguez et al., 2006). Most anaerobic fermentation models are based on a kinetic analysis, in which the stoichiometry is considered to be constant (Aceves-Lara et al., 2008). In many batch or continuous experiments the stoichiometry of the biochemical reactions in MCF has been shown to depend on the environmental conditions such as pH, substrate type, substrate concentration, and so on (Fang and Liu, 2002; Kim et al., 2006; Temudo et al., 2007). A variable stoichiometry is clearly necessary in anaerobic fermentation modeling.

A metabolic model with variable stoichiometry of MCF was proposed by Rodriguez et al. (2006) (hereafter termed Rodriguez model) based on the following assumptions: a

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mixed culture under carbon and energy-limited conditions will strive for maximum catabolic energy generation or maximum growth yield; the optimization is constrained by mass balances and thermodynamics of the bioreactions involved; the most common fermentative pathways, including acetate, propionate, butyrate, and lactate producing, are considered; $NADH/NAD^+$ is the only redox-couple considered to produce H_2, ethanol and butyrate; a proton gradient over the cytoplasmic membrane is generated by the excretion of undissociated acidic products, undissociated acids in the bulk solution lead to energy dissipation of the membrane potential (Rodriguez et al., 2006). The model was used to predict the distribution of metabolic products in an anaerobic fermentation: ethanol was suggested as the main product at low pH (<5.5); acetate dominated at low hydrogen pressure and neutral pH (6–9), while butyrate became the major product at intermediate pH (5.5–6) and/or higher hydrogen pressure. However, most experimental results show butyrate and acetate are main products at acidic pH (4–6.5), while acetate, ethanol, and/or propionate appear at neutral and alkaline pH (6.25–8.5; Fang and Liu, 2002; Kim et al., 2006; Temudo et al., 2007). The mismatch between prediction and experimental results significantly limits the model application and urges further model development.

Two important assumptions of Rodriguez model, NADH being used for H_2 production and the energy consumed for organic acid transport, are considered not properly incorporated in the model. Ferredoxin (Fdred) is the general electron carrier for the reduction of intracellular H^+ to H_2 (Temudo et al., 2007). The potential of Fdred/Fdox (−400 mV, or a little lower; Thauer et al., 2008) is much closer to H_2/H^+ (−414 mV) than that of $NADH/NAD^+$ (−320 mV), Fdred is the suitable electron donor for H^+ reduction at hydrogen partial pressure above 60 Pa as has been verified for the hydrogen formation by *T. tengcongensis* (Soboh et al., 2004). For fermentative conditions a main question is how to balance the extra NADH when it is not used for hydrogen production. The conversion of NADH to Fdred could solve an intracellular NADH unbalance (Lee et al., 2009), but this reaction is thermodynamic unfavorable. Energy conserving electron bifurcation reaction would be one solution to resolve this puzzle. Energy conserving electron bifurcation reaction is defined as the energy generated from an exergonic reaction driving the endergonic reaction by a functional enzyme complex, for example, butyryl-CoA production from crotonyl-CoA by NADH oxidation drives the endergonic reaction of Fdox reduction by NADH oxidation (Equation 1; Herrmann et al., 2008). Schut et al. found almost one-third of the known H_2-producing anaerobes could simultaneously produce H_2 from NADH and Fdred through an energy conserving electron bifurcation reaction with the ratio at approximate 1:1 by a trimetric bifurcating enzyme (Equation 2). Moreover, energy conserving electron bifurcation reaction has already been verified in the production of butyrate (Seedorf et al., 2008) and methane (Thauer et al., 2008), and so on

$$\text{Crotonyl} - \text{CoA} + \text{Fdox} + 2\text{NADH} \rightarrow \text{Butyryl} - \text{CoA} + \text{Fdred} + 2\text{NAD}^+ \quad (1)$$

$$\text{Fdred} + \text{NADH} + 3\text{H}^+ \rightarrow \text{Fdox} + \text{NAD}^+ + 2\text{H}_2 \quad (2)$$

The inwards diffusion of organic acids over the cytoplasmic membrane leads to dissipation of the proton motive force since the compound has to be transported out of the cell again. The proton motive force (pmf) combined with a transport rate coefficient of acidic products have been proposed as factors for modeling this energy loss (Rodriguez et al., 2006; Russell and Diez-Gonzalez, 1997). Firstly, the pmf has been considered to be dissipated by the undissociated organic acid gradient over the cytoplasmic membrane (Rodriguez et al., 2006). However, this assumption is doubtable. The pmf is the sum of proton gradient and electron potential difference of a cytoplasmic membrane. For the cellular homeostasis, a microbe would realize the interconversion of proton gradient and electron potential difference under different environmental conditions, which consumes energy in the form of 1/3, 1/4, or 1/5 ATP (Dimroth and Cook, 2004; Maris et al., 2004; Stams and Plugge, 2009). Because the export of metabolic organic acid by membrane-bound proteins shows a higher efficiency (Lehninger et al., 2005), the membrane-bound ATPase relevant to pmf is considered to expel the H^+, and the organic anions are exported with symport system (Abbott et al., 2007; Maris et al., 2004). So the pmf utilization for organic acid transport can be assumed as constant around the spectrum of pH (even at alkaline conditions) and temperature. Secondly, the difference will be the diffusion rate of organic acids into the cell. The exact diffusivity parameters of undissociated acids over the cell membrane are not available till now. The data used in the Rodriguez model are rough estimates. Thereby, it is necessary to re-evaluate them under constant pmf.

Here we adopt the Rodriguez model with above-mentioned aspects, besides we also add a few smaller changes that will be introduced below. Finally the modeling results of MCF from glucose are presented and discussed to check whether the modifications give a more realistic description of experimental results.

The Modified Metabolic Model for Mixed Culture Fermentation

Our metabolic model is a modification from Rodriguez model which is based on mixed culture glucose fermentation in a continuous stirred tank reactor (CSTR) under stable environmental conditions. The basic constraints of the model are mass balance, redox balance, and energy balance.

The maximum energy generated (in the form of ATP) as the function of metabolites from glucose is assumed in our model, but is considered with the fundamental physico-chemical constrains and conditions-dependent environmental constraints.

The Metabolic Pathways of Glucose Fermentation—Modeling Foundation

The metabolic pathways of anaerobic glucose fermentation are shown in Figure 1. Energy conservations are considered to occur via substrate level phosphorylation (SLP) in catabolic reaction, ion motive force (Biegel et al., 2011), and energy conversation electron bifurcation reaction (Herrmann et al., 2008). The redox-couples are NADH/NAD^+ and Fdred/Fdox. The main metabolites are acetate, butyrate, propionate, lactate, ethanol, H_2, and CO_2. Dotted lines are new pathways due to the involvement of energy conserving electron bifurcation reaction, Fdred/Fdox, etc., which will be described in details below. The formation reactions of pyruvate, acetate, ethanol, lactate, biomass, and ATP are the same as in the Rodriguez model, which are described in Supplementary Information.

Butyrate ($C_4H_7O_2^-$) Formed From Pyruvate

The formation of butyrate can occur by two different pathways or enzyme-groups, a combination of phospho-transbutyrylase and butyrate kinase (Equation 3), and butyryl-CoA:acetate CoA-transferase (Equation 4). The latter pathway was introduced in the Rodriguez model. However, biochemical studies from human colon bacteria show that the first pathway is the primary pathway for butyrate production (Equation 3) at low concentration of acetate, while at higher acetate concentration (30–80 mM), the conversion switches to the second pathway (Equation 4) to decrease the toxicity of acetate though the energy yield is lower (0 ATP; Louis et al., 2004). The high acetate concentration pathway (Equation 4) is not considered in our model. Furthermore, the reduction of crotonyl-CoA to butyryl-CoA by NADH is assumed to be related to an energy conserving electron bifurcation reaction with Fdred produced (Seedorf et al., 2008)

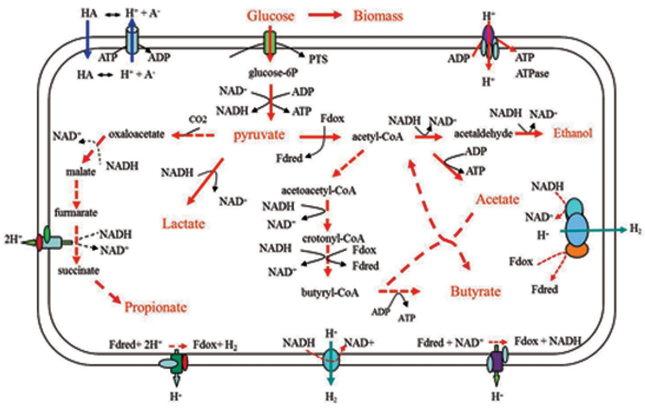

Figure 1. The metabolic pathways of mixed culture glucose fermentation adapted from textbooks (Gottschalk, 1986; Lehninger et al., 2005; Lengeler et al., 1999). Dotted lines represent the modified pathways.

$$2C_3H_3O_3^- + 3\text{Fdox} + 3\text{NADH} + \text{ADP} + \text{Pi} \rightarrow C_4H_7O_2^- + 2CO_2 + 3\text{Fdred} + 3NAD^+ + H^+ + H_2O + \text{ATP} \quad (3)$$

$$C_3H_3O_3^- + C_2H_3O_2^- + 2\text{Fdox} + 3\text{NADH} \rightarrow C_4H_7O_2^- + 2\text{Fdred} + 3NAD^+ + CO_2 + H_2O \quad (4)$$

Propionate ($C_3H_5O_2^-$) Formed From Pyruvate

The methylmalonyl-CoA pathway is considered to be the pathway for propionate production in the MCF model with 2/3 ATP yield, which is considering the strive for maximum yield and the effect of higher CO_2 concentration at neutral and alkali pH (Seeliger et al., 2002; Stams and Plugge, 2009)

$$C_3H_3O_3^- + 2\text{NADH} + \frac{8}{3}H^+ + \frac{2}{3}\text{ADP} + \frac{2}{3}\text{Pi} \rightarrow C_3H_5O_2^- + 2NAD^+ + \frac{2}{3}\text{ATP} + \frac{5}{3}H_2O \quad (5)$$

Hydrogen (H_2) Formed Form NADH and Fdred

Hydrogen production with energy conserving electron bifurcation reaction is adapted from Schut and Adams (2009) with some modification: at lower hydrogen pressure, the hydrogen is produced form both Fdred (1/3 ATP is generated; Sapra et al., 2003) and NADH (Equations 6 and 7), respectively. In the intermediate concentration range, hydrogen is produced simultaneously from Fdred and NADH, but partially NADH is also used to the reduction reaction (Equation 8); at higher hydrogen pressure, hydrogen is produced only from Fdred (Equation 6, without ATP yield), part of Fdred is also used to the reduction of NAD^+ (Equation 9). Therefore, the pathway of hydrogen production is very different from that in the Rodriguez model, in which NADH is the sole redox

couple for H_2 production

$$\text{Fdred} + \left(2 + \frac{1}{3}\right)\text{H}^+ + \frac{1}{3}\text{ADP} + \frac{1}{3}\text{Pi} \rightarrow \text{Fdox} + \text{H}_2 + \frac{1}{3}\text{ATP} + \frac{1}{3}\text{H}_2\text{O} \quad (6)$$

$$\text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+ + \text{H}_2 \quad (7)$$

$$\text{Fdred} + \text{NADH} + 3\text{H}^+ \rightarrow \text{Fdox} + \text{NAD}^+ + 2\text{H}_2 \quad (8)$$

$$\text{Fdred} + \text{NAD}^+ + \text{H}^+ \rightarrow \text{Fdox} + \text{NADH} \quad (9)$$

Other metabolites production, such as butanol, valerate, caproate, acetone, and glycerol, are not considered at this stage. In mixed culture fermentations they usually play only a minor role. The lumped pathway (from Equations 3–9 and Supplementary Equations S1–S5, and from v1 to v13 in Supplementary Fig. S1) with metabolites transport (from t1 to t11 in Supplementary Fig. S1) and algebraic network (Supplementary Fig. S2) can be found in Supporting Information. The total number of flux equations is 24. After applying mass balance and metabolic pathway constraints, it comes out 6° of freedom as shown in supporting information.

The Constraints of the Model

Thermodynamic Constraints

The biochemical reactions are constrained by thermodynamic control. The standard Gibbs free energy of formation and standard enthalpy of formation for the relevant compounds are shown in Table I (Kleerebezem and Van Loosdrecht, 2010; Lee et al., 2008; Smith et al., 1995). The calculation of the reaction of Gibbs free energy can refer to other researchers (Alberty, 2003; Speight and Lange, 2005).

Energy Involved in Metabolites Transport Across Cytoplasmic Membrane (ATP_{Tr})

The transport of metabolites requires energy primary present as proton motive force (pmf). The pmf gives the energy for ATP generation by the membrane-bound ATPase. For the efflux of metabolites also pmf-derived energy is used (Fig. 1). This energy is equivalent to the rate of passive transport by diffusion of metabolites from the bulk liquid into the cell. The pmf is considered to be constant in our model with 1/4 ATP consumed per H^+ exported (Maris et al., 2004). The calculation of energy used for metabolites transport is shown in Equation (10), in which, V_i is the production rate of metabolic acid, mol/(L h); Diff_{AH} is the transport rate coefficient, L/(mol_x h); $C_{\text{AH},e}$ and $C_{\text{AH},i}$ are the concentrations of undissociated organic acid, mol/L; X is the concentration of biomass, mol_x/L. The constant pmf energy (1/4 ATP per proton) decreases the complexity of the initial estimation for the optimal flux, which results in the change from nonlinear (Rodriguez model) to linear optimization systems in our model, as shown in Supporting Information

$$\text{ATP}_{\text{tr}} = \frac{1}{4}(V_i + \text{Diff}_{\text{AH}}(C_{\text{AH},e} - C_{\text{AH},i})X) \quad (10)$$

Energy Consumed for Maintenance (ATP_m)

The calculation of energy consumed for maintenance (E_{m}, kJ/(mol_x h)) is proposed by Tijhuis et al. (1993). However, the values calculated are far higher than that of real fermentative systems, even in an order of magnitude (Adams et al., 2006). The equation is modified with the glucose data collected by Tijhuis, as shown in Equation (11), in which R is 8.314 J/(mol K); T is the temperature, K. The E_{m} calculated from Equation (11) is 3.79 kJ/(mol_x h), which is comparable to 5.24 kJ/(mol_x h) adopted by Rodriguez model. But, this equation would allow the use of the model at higher temperature, which is under research

$$E_{\text{m}} = 3.79\exp\left(\frac{4.74 \times 10^4}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right) \quad (11)$$

The ATP consumed for maintenance (ATP_{m}) is shown in Equation (12), where E_{ATP} is the energy consumed for ATP synthesis (kJ/mol):

$$\text{ATP}_{\text{m}} = \frac{E_{\text{m}}}{E_{\text{ATP}}} \quad (12)$$

Table I. The $\Delta G_{\text{f}}^{\circ}$ and $\Delta H_{\text{f}}^{\circ}$ of the fermentation metabolites.

Metabolite	State	$\Delta G_{\text{f}}^{\circ}$ (kJ/mol)	$\Delta H_{\text{f}}^{\circ}$ (kJ/mol)
Glucose	Aqueous	−915.90	−1262.19
Pyruvate	Aqueous	−472.27	−596.22
Acetate	Aqueous	−369.31	−486.01
Butyrate	Aqueous	−352.63	−535.55
Ethanol	Aqueous	−181.64	−288.3
Propionate	Aqueous	−354.80	−510.80
Lactate	Aqueous	−516.72	−686.64
Pi	Aqueous	−1096.1	−1299
ADP	Aqueous	−1906.13	−2626.54
ATP	Aqueous	−2768.10	−3619.21
NAD^+	—	0	0
NADH	—	22.65	−31.94
Fdred[a]	—	0	—
Fdox[a]	—	−77.20	—
CO_2	Gas	−394.36	−393.50
H_2	Gas	0	0
H_2O	Liquid	−237.19	−285.83
NH_3	Aqueous	−26.50	−80.29
Proton	Aqueous	0	—
Biomass	Solid	−67	−91

[a]The relative potential of Fdred/Fdox is adapted from Smith et al. (1995).

Parameters for the Metabolic Model

The parameters used for the metabolic model of mixed culture glucose fermentation include physiological parameters, the criteria for hydrogen production, and the energy consumed for metabolites transport.

Physiological Parameters

The physiological parameters are variable among species of microorganisms, and even change with environmental conditions (Maskow and Stockar, 2005). The parameters used in the model are adapted from the Rodriguez model with modification for the transport rate coefficient (Table II).

The Criteria of Hydrogen Production

The stoichiometry of hydrogen has already been proposed above, however the criteria of the three stages have not been defined yet. These stages are related to temperature, ΔG_{min}, and the energy involved. To clarify this assumption, the redox couple of NADH/NAD^+ and Fdred/Fdox could be calculated as follows.

Stage 1(S_1): Low Hydrogen Partial Pressure

The calculation of Fdred/Fdox and NADH/NAD^+ is derived from Equations (6) and (7), as shown in Equations (13) and (14); P_{H_2} is the partial pressure of hydrogen, atm/atm; 10^{pHin} is the intracellular concentration of H^+, mol/L; ΔG_{Eq} is the Gibbs free energy of reaction, kJ/mol; ΔG_{min} is the minimum energy of biochemical reactions, kJ/mol

$$\frac{\text{Fdred}}{\text{Fdox}} = P_{H_2} \times (10^{pHin})^2 \exp\left(\frac{\Delta G_{Eq.\,(6)} - \Delta G_{min}}{RT}\right) \quad (13)$$

$$\frac{\text{NADH}}{\text{NAD}^+} = P_{H_2} \times 10^{pHin} \exp\left(\frac{\Delta G_{Eq.\,(7)} - \Delta G_{min}}{RT}\right) \quad (14)$$

Table II. The physiological parameters used in our metabolic model.

Parameters	Value	Unit
pH_{in}	7.0	—
C^i_{Pyr}	7.5	mM
C_{ATP}	7.9[a]	mM
C_{ADP}	1.04[a]	mM
C_{Pi}	7.9[a]	mM
$maxC^i_{Ac}$	10	mM
$maxC^i_{Bu}$	10	mM
$maxC^i_{Pro}$	10	mM
$maxC^i_{Lac}$	10	mM
$maxC^i_{EtOH}$	10	mM
$Diff_{AcH}$	30	1/(M × h)
$Diff_{AcH}$	123[b]	1/(M × h)
$Diff_{BuH}$	28	1/(M × h)
$Diff_{BuH}$	85[b]	1/(M × h)
$Diff_{ProH}$	106	1/(M × h)
$Diff_{ProH}$	100[b]	1/(M × h)
$Diff_{LacH}$	15	1/(M × h)
$Diff_{LacH}$	100[b]	1/(M × h)
ΔG_{min}	−5	kJ/mol

[a]The values are adapted from Maskow and Stockar (2005).
[b]The transport rate coefficients are adapted from Rodriguez model.

Stage 2 (S_2): Middle Hydrogen Partial Pressure

To calculate the ratio of NADH/NAD^+ and Fdred/Fdox, respectively, Equation (8) should be divided to Equations (15) and (16), with 1/4 ATP is assumed for the interconversion (Seedorf et al., 2008; Thauer et al., 2008). The ratios of Fdred/Fdox and NADH/NAD^+ are derived from Equations (17) and (18), respectively

$$\text{Fdred} + \left(\frac{1}{4} + 2\right)\text{H}^+ + \frac{1}{4}\text{ADP} + \frac{1}{4}\text{Pi} \rightarrow \text{Fdox} + \text{H}_2 + \frac{1}{4}\text{ATP} + \frac{1}{4}\text{H}_2\text{O} \quad (15)$$

$$\text{NADH} + \text{H}^+ + \frac{1}{4}\text{ATP} + \frac{1}{4}\text{H}_2\text{O} \rightarrow \text{NAD}^+ + \text{H}_2 + \frac{1}{4}\text{ADP} + \frac{1}{4}\text{Pi} + \frac{1}{4}\text{H}^+ \quad (16)$$

$$\frac{\text{Fdred}}{\text{Fdox}} = P_{H_2} \times (10^{pHin})^2 \exp\left(\frac{\Delta G_{Eq.\,(15)} - \Delta G_{min}}{RT}\right) \quad (17)$$

$$\frac{\text{NADH}}{\text{NAD}^+} = P_{H_2} \times 10^{pHin} \exp\left(\frac{\Delta G_{Eq.\,(16)} - \Delta G_{min}}{RT}\right) \quad (18)$$

Stage 3 (S_3): High Hydrogen Partial Pressure

The calculation of Fdred/Fdox (Equation 19) and NADH/NAD^+ (Equation 20) are derived from Equations (6) and (9), respectively

$$\frac{\text{Fdred}}{\text{Fdox}} = P_{H_2} \times (10^{pHin})^2 \exp\left(\frac{\Delta G_{Eq.\,(6)} - \Delta G_{min}}{RT}\right) \quad (19)$$

$$\frac{\text{NADH}}{\text{NAD}^+} = \frac{\text{Fdred}}{\text{Fdox}} \times 10^{pHin} \exp\left(\frac{\Delta G_{min} - \Delta G_{Eq.\,(9)}}{RT}\right) \quad (20)$$

Criteria of the Three Stages Selection

It is assumed that, the maximum yield of H_2 would be 4, 3, and 2 mol/mol_Glucose at S_1, S_2, and S_3, respectively; while

the residual NADH and Fdred would be consumed to produce ethanol, propionate, etc. (Schut and Adams, 2009; Soboh et al., 2004). Because the ratio of Fdred/Fdox is not available in the literature, while the values of NADH/NAD ratio have been reported in several papers (Garrigues et al., 1997), we propose that the criteria to identify the three stages is solely related to the ratio of $NADH/NAD^+$, which can be described as $S_1 < H_a < S_2 < H_b < S_3$. The partition value H_a for S_1 and S_2 is assumed to be 0.05 molNADH/molNAD (Garrigues et al., 1997). And, the partition value H_b (molNADH/molNAD) for S_2 and S_3 (calculated from Equation 20) is evaluated in this study (see Results Section).

Energy Consumed for Metabolite Transport

Exporting the metabolic undissociated acids across cytoplasmic membrane consumes an amount of energy related to the membrane-bound ATPase. The total energy for any metabolite can be calculated with the sum of energy yield by SLP and consumed for transport, as shown in Equation (21) in the form of ATP per mol_glucose consumed. The ATP_{Tr} is derived from Equation (10), the detailed calculation is given in Supporting Information. It should be noted that the modified metabolic model is optimized on the maximum energy generated (in the form of ATP) as function of metabolites from glucose. Therefore, as each energy of metabolites is calculated from Equation (21), a linear optimization routine could be used

$$E_{ATP}^{net} = E_{ATP}^{yield} - E_{ATP}^{Tr} \quad (21)$$

At acidic pH, metabolic acids (such as acetic, butyric acid) act as the uncoupling agents, which are toxic to the bacteria (Maris et al., 2004; Nakano et al., 2006) and lead to a metabolic pathway shift (Rodriguez et al., 2006). However, the exact transport rate coefficient is not available till now. The effect of transport rate coefficients on metabolites distribution would be discussed below.

Modeling Results in a Continuous Stirred Tank Reactor (CSTR)

The scheme of model evaluation (see Supplementary Fig. S3 which is based on the algebraic network in Supplementary Fig. S2 and the constrained conditions from Equations 10–21) is modified from the Rodriguez model. First a biomass yield is proposed, then the optimum criterion is applied to calculate the metabolite yield and the optimized biomass under physiological and thermodynamic evaluation with MATLAB 7.8.0 (2009a). The modification of the scheme is the involvement of the criteria for hydrogen production before the optimum calculation. The metabolic model is used to model glucose fermentation in a CSTR reactor under the following conditions: 22.2 mM glucose concentration (except stated in the text), pH 5.5 (except stated in the text), temperature at 303.15 K and HRT at 16 h.

Evaluation of Partition Value H_b

The partition between intermediate and high cellular NADH/NAD ratio (H_b value) is evaluated for different pH values whereas the transport rate coefficients of metabolites are adopted from the Rodriguez model. As shown in Figure 2A, there is no significant difference of hydrogen yield between a partition value of (H_b) 1 or 2, the hydrogen yield is about 2.2 mol/mol_glucose in the pH range of 5.5–6 and decreases to 0.7 mol/mol_glucose under neutral and alkaline pH (7–9). By increasing the partition value to 4, the H_2 yield is about 2 mol/mol_glucose under neutral and alkaline pH, which is far away from the literature data (below 1 mol/mol_glucose; Lee et al., 2009; Temudo et al., 2007). Furthermore, as shown in Figure 2B, under pH 5.5, the hydrogen partial pressure for S_2 increases from 0.16

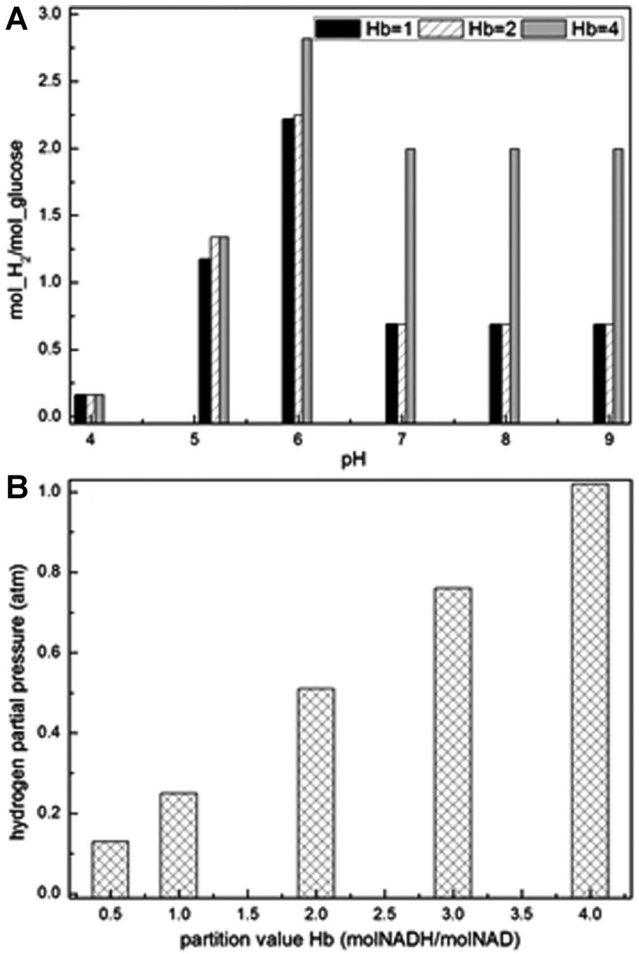

Figure 2. The effect of partition value for intermediate and high hydrogen partial pressure (H_b) on the H_2 yield (**A**) and hydrogen partial pressure (**B**).

to 1.0 atm as partition value H_b increases from 0.5 to 4.0. As proposed above, the maximum hydrogen yield at S_2 would be 3 mol/mol_glucose. The hydrogen yield from the literature is 2 mol/mol_glucose at the hydrogen partial pressure above 0.5 atm, and it increases slightly (below 3 mol/mol_glucose) at the hydrogen pressure below 0.5 atm (Kim et al., 2006; Temudo et al., 2007; Van Ginkel and Logan, 2005a). Therefore, the partition value H_b of 1 is applied in our model.

The three-stages of hydrogen production could control the interconversion of NADH/NAD^+ and Fdred/Fdox and regulate metabolic distribution in the view of redox balance. That is, at S_2, a fraction of produced NADH can produce H_2 with Fdred in the energy conserving electron bifurcation reaction, the remainder of NADH is consumed to generate ethanol, lactate, propionate, etc.; at S_3, all NADH will be consumed to generate reduced metabolites, such as ethanol. It shall be clarified that the hydrogen yield (2.2 mol/mol_glucose) in Figure 2A is higher than the maximum yield (2 mol/mol_glucose) set for S_3 in our model. This is because the energy conserving electron bifurcation reaction is adopted for both hydrogen and butyrate. NADH is reduced to generate Fdred in the production of butyrate even at higher hydrogen pressure (S_3), which leads to a little higher hydrogen yield.

Effect of Transport Rate Coefficients on Metabolic Distribution

As shown in Equations (10) and (21) and Supporting Information, the transport rate coefficients are related to the energy yield from catabolic reactions, which can affect the distribution of metabolites: the lower transport rate coefficient (i.e., less cell-inward diffusion of metabolites) results in higher net energy yield. Using the transport rate coefficients in the Rodriguez model, the main metabolites are ethanol at pH below 5, butyrate and acetate between 5.3 and 6.0, acetate and propionate above 6.0, respectively (Fig. 3A). However, the main metabolites from the literature are butyrate and acetate, not ethanol when pH is below 5 (Fang and Liu, 2002; Temudo et al., 2007). The inconsistent model results are mainly due to the relative higher transport rate coefficients of the Rodriguez model for compounds with a low octanol/water distribution coefficient.

With the modified transport rate coefficients in Table II, the results of our model are much closer to the literature data: ethanol, acetate, and butyrate at pH below 6.0, acetate and propionate at pH above 6.0, respectively (Fig. 3B). The hydrogen yield in Figure 3B is also comparable to the literature (Fang and Liu, 2002; Lee et al., 2008; Temudo et al., 2007). Biomass yields are similar in both approaches, about 1.0 mol/mol_glucose. Especially, as shown in Figure 3C, under the same experimental conditions, our model can reflect the changing of metabolites from the literature (Fang and Liu, 2002). These results indicate that the proposed transport rate coefficients in Table II are good

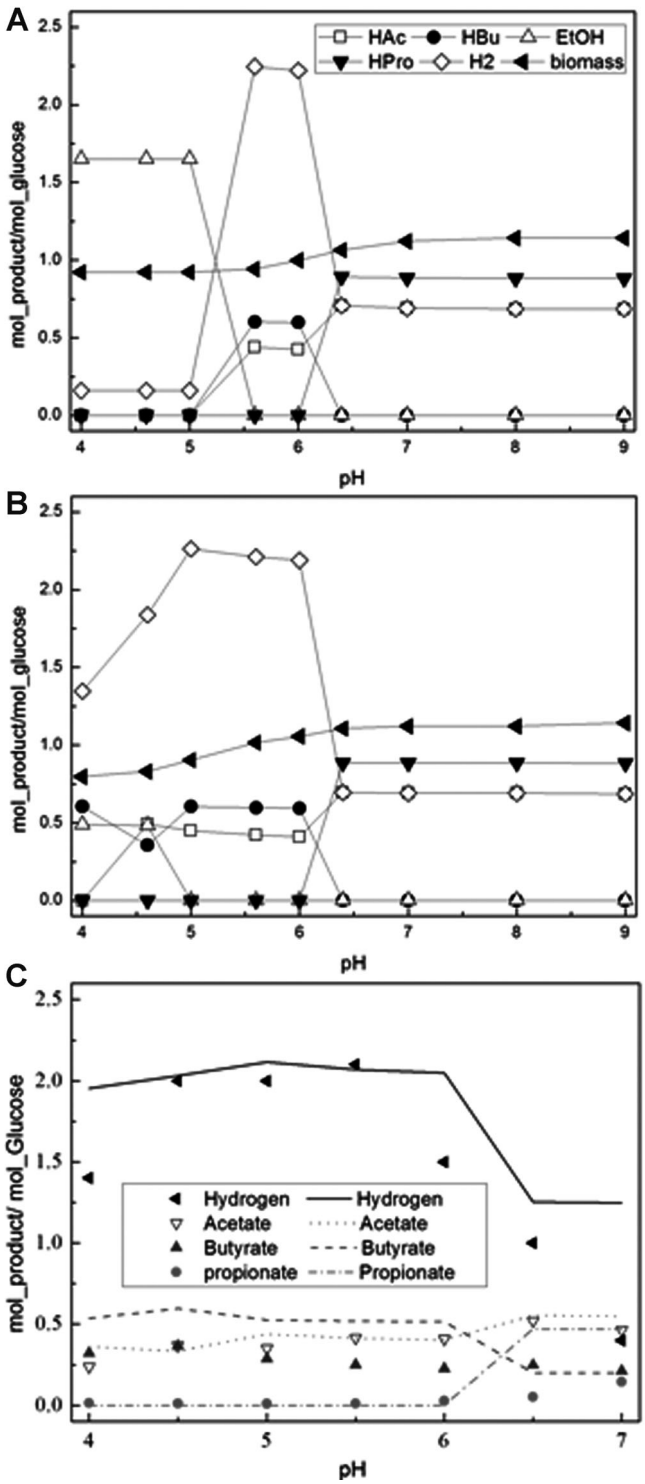

Figure 3. The effect of transport rate coefficients on the yields of metabolites under different pH from Rodriguez model (**A**), our model (**B**), and the literature (**C**) notes: in C, scatter symbols corresponded to data from Fang and Liu (2002), line indicated the model results.

estimates. It shall be noted that the experimental data in Figure 3C are adapted from Fang and Liu (2002), in which methane was detected at pH between 6.0 and 7.0. The

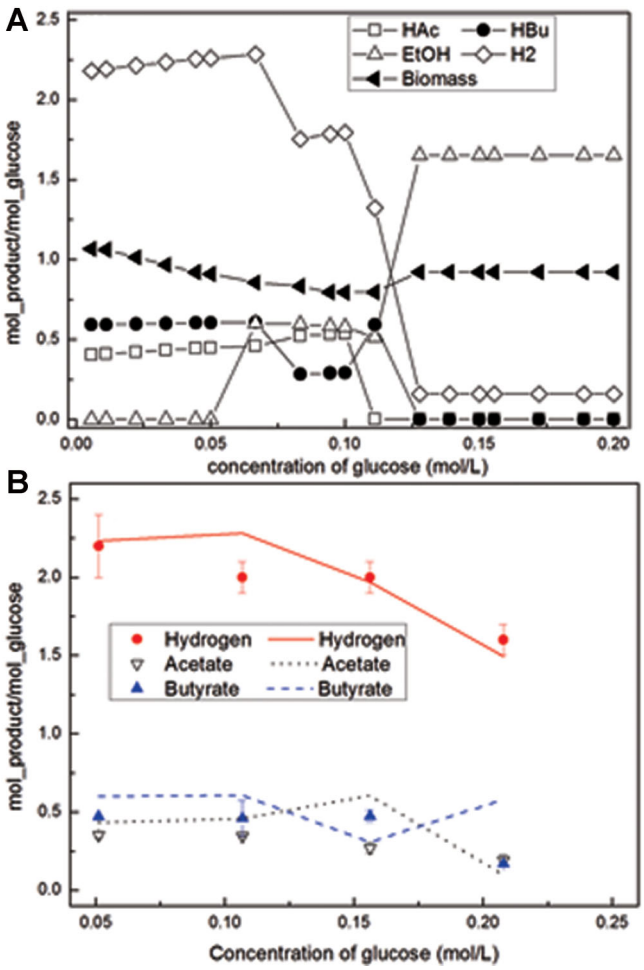

Figure 4. The metabolic yields under different concentration of influent glucose (**A**) and comparison with the literature (**B**) notes: in B, scatter symbols corresponded to data from Van Ginkel and Logan (2005a).

methane production likely led to the lower hydrogen yield. Therefore, the hydrogen yield in our model is a little higher than that of experimental data.

Effects of Influent Substrates Concentration on Metabolic Distribution

Figure 4A shows the effects of influent substrate concentration on metabolite yields at pH 5.5. When influent glucose concentration is below 0.06 mol/L, the main metabolites are acetate and butyrate, and the hydrogen yield is 2.2 mol/mol_glucose. When glucose is above 0.06 mol/L, ethanol is generated, butyrate and hydrogen decrease to 0.3 and 1.8 mol/mol_glucose, while the yield of acetate does not change significantly. At higher glucose concentration (above 0.13 mol/L), the main metabolites shift to ethanol, associated with a little hydrogen production (for the

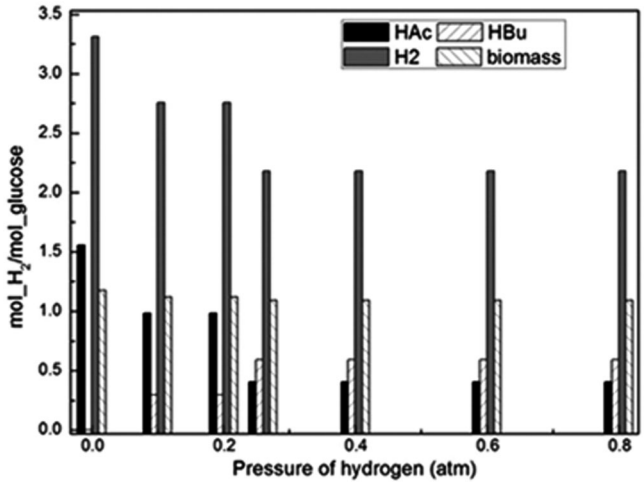

Figure 5. The metabolic yields under different hydrogen partial pressure.

formation of NADH coinciding with biomass). The biomass yields do not change much at different glucose concentrations. As shown in Figure 4B, our modeling results can reflect the change of metabolites under different glucose concentrations from the literature (Van Ginkel and Logan, 2005b).

Effects of Hydrogen Partial Pressure on Metabolic Distribution

The effect of hydrogen partial pressure on metabolite yields at pH 5.5 is illustrated in Figure 5. It can be seen that our model can predict the changes of acetate and butyrate with the increasing hydrogen partial pressure, that is, at 1E−5 atm of H_2 pressure, the main metabolites are acetate and hydrogen (3.3 mol/mol_glucose); the hydrogen yield decreases to 2.7 mol/mol_glucose till 0.2 atm of H_2 pressure, butyrate starts to be generated at the same time. Furthermore, as H_2 pressure increases to 0.4 atm, the yields of hydrogen and acetate decrease to 2.2 and 0.4 mol/mol_glucose respectively, and the butyrate yield increases to 0.7 mol/mol_glucose, which is consistent with the literature (van Andel et al., 1985). In Figure 5, the biomass yields do not change much at different hydrogen partial pressures and no ethanol or propionate is generated.

However, it shall be noted that the hydrogen yield is a little higher than mentioned in some publications (Kim et al., 2006; Temudo et al., 2007). For example, the hydrogen yield was just 1.65 mol/mol_glucose when N_2 sparging rate was 120 mL/min (Temudo et al., 2007). And, the hydrogen yield just increased from 0.77 to 0.92 mol/mol_glucose as the hydrogen partial pressure decreased from 63.2% to 3.9% in another experiments (Kim et al., 2006). As known, hydrogen supersaturation is common in mesophilic

fermentation reactor, which was much higher under sparging conditions (Kraemer and Bagley, 2006). For example, the value of supersaturation ratio increased from 3 to 11 when N_2 sparging rate was 160 mL/min. Therefore, the hydrogen content in liquid is the more suitable factor and will be involved in future work.

Discussion

In our mixed culture fermentation model, the redox couples for hydrogen production (S_1, S_2, S_3) are more complex than in the Rodriguez model, and it can efficiently regulate the redox balance and control the distribution of metabolites. Especially, the energy conserving electron bifurcation reaction, which is a recent recognized aspect of fermentative metabolic pathways, is adopted in the stoichiometry of hydrogen. It is reported that almost one-third of the known H_2 producing bacterial species, which contain a homolog of the catalytic subunit of [FeFe] hydrogenases, may have the potential mechanistic function of energy conserving electron bifurcation reaction in hydrogen production (Schut and Adams, 2009). Also the hydrogen yield, which is higher than 2 mol/mol_glocose in some experiments, also supports this metabolic pathway (Sieber et al., 2010). Furthermore, this concept could benefit for other puzzled and unclear metabolic pathways (Thauer et al., 2008) and would be also considered to other metabolic models to guide the study of metabolism in turn. For example, Herrmann et al. (2008) and Li et al. (2008) demonstrated butyrate production involved energy conserving electron bifurcation reaction with Fdred formed. And this pathway also was proposed on the study of human colon bacteria (Louis and Flint, 2009). For propionate formation, acryloyl-CoA pathway is not used in our model as it is beneficial for the faster specific substrate turnover (Seeliger et al., 2002) but with low energy yield. However, it is also suspected that the energy conserving electron bifurcation reaction may involve in the reduction of acryloyl-CoA to propionyl-CoA (Herrmann et al., 2008).

It is well known that, membrane-bound ATPase is essential to bacteria and yeasts (Dimroth and Cook, 2004). In return, metabolites transportation by those ATPase would affect the metabolic fluxes and growth yield. For example, the conversions of glucose to lactate and ethanol yield the same ATP, but *Saccharomyces cerevisiae* (a beer yeast) could not sustain in the homolactate fermentation for exporting lactic acid at low pH (Abbott et al., 2009); while, homolactate fermentation is the main pathway by lactic acid bacteria (even pH below 4; Lengeler et al., 1999). The different performance between *S. cerevisiae* and lactic acid bacteria is due to 1 ATP and 1/4 ATP required for the lactic acid export, respectively, resulting in *S. cerevisiae* preferring ethanol production as it does not require transporting ATP for ethanol (Maris et al., 2004).

Until now, the exact transport rate coefficient of metabolites are not available (Rodriguez et al., 2006), but these data are important for metabolites distribution in the view of maximum energy generated. For bacteria (such as *Ethanologenic Escherichia coli LY01*) and *yeasts*, the octanol–water partition coefficient is an important criterion to determine the toxicity and diffusivity, so the order of transport rate coefficient is lactate < acetate < propionate < butyrate (Abbott et al., 2007). But this tendency is contrary to some values used in the Rodriguez model (the order of transport rate coefficient is butyrate < lactate = propionate < acetate). Besides, the cell would change the composition to adapt the environmental response, such as pH, temperature, and toxicity, etc. (Aguilar and De Mendoza, 2006; Chhabra et al., 2006). So, it is putative that the transport rate coefficients proposed in our model (i.e., lactate < butyrate < acetate < propionate) meet the biochemical and biophysical character of mixed culture fermentation of glucose.

Recently, some studies found that pH_{in} may change with environmental conditions to keep the homeostasis of microorganisms, especially with pH_{out} (Russell and Diez-Gonzalez, 1997). However, there is no general information in the literature showing the relation between pH_{in} and pH_{out}. Therefore a constant intracellular pH is considered in this model. Since the pH difference over the cytoplasmic membrane could significantly affect the metabolic distribution, the changing pH_{in} should be evaluated in future works provided that an intrinsic function between pH_{in} and pH_{out} becomes also available. On the other hand, the presented metabolic model is a modification from the Rodriguez model, in which, the most common catabolic reactions known for glucose fermentation were considered (Rodriguez et al., 2006). The functional communities in mixed culture fermentation are normally dominated by *Clostridium* sp., and our modified model is suitable to simulate their metabolic diversity. For other type of fermentations, such as lactate-type fermentation, the produced energy is lower than that for acetate and butyrate fermentations, our model cannot directly simulate lactate fermentation. An additional switching function could be used to include other specific strain fermentation pathways.

Finally, it should be recommended that the model here is based on the biochemical and biophysical concept with thermodynamic constraint. However, it is shown that the metabolic reactions are jointly controlled by the enzyme capacity and metabolic concentration (Fendt et al., 2010). As proposed by Kotte et al. (2010), both enzyme kinetic and transcriptional regulation are involved to model the metabolic operation between glycolytic and gluconeogenic of *E. coli* instead of Boolean calculation (i.e., AND, OR, NOT). So, the coupling of thermodynamic and enzyme kinetic with some constraint equations (such as ion strength; Niel et al., 2003) will be the orientation of the future MCF study. Due to the complexity of the MCF model, more elaborated work is needed to study and adjust the parameters, including values of the criteria for hydrogen production, transport rate coefficient, other metabolic

pathways such as the formate/hydrogen lyase conversion, etc. Meanwhile, similar to the Rodriguez model, since our modified model illustrates an improved methodology for estimating the metabolic stoichiometry of glucose in mixed culture fermentation, it can be applied on other fermentation strategies, such as batch and UASB systems.

Conclusions

The here proposed modified metabolic model for mixed culture fermentation has an improved alignment with experimental results from the literature: (1) pH effect: butyrate, acetate, and ethanol are produced at acidic pH (4–6.0), while acetate and propionate are produced at neutral and alkali pH, the hydrogen production is lower than 2.2 mol/mol_Glucose; (2) glucose concentration effect: at high glucose concentration (above 0.13 mM), ethanol becomes the main metabolite with low hydrogen yield; (3) hydrogen partial pressure effect, the changes of acetate and butyrate is following with increasing hydrogen partial pressure. Further development of this model structure will benefit the development of mixed culture fermentations with the aim of producing specific products.

Nomenclature

MCF	mixed culture fermentation
pmf	proton motive force
Fdred	reluctant Ferredoxin
Fdox	oxidant Ferredoxin
NADH	Nicotinamide adenine dinucleotide
pH_{in}	intracellular pH
pH_{out}	extracellular pH
X	concentration of biomass (mol/L)
$Diff_{AH}$	the transport rate coefficient ($L/(mol_x\,h)$)
ATP_{Tr}	ATP consumed for metabolic organic acid exporting, $mol_{ATP}/(mol_x\,h)$
E_m	Gibbs energy consumed for maintenance, $kJ/(mol_x\,h)$
ATP_m	ATP consumed for maintenance, $mol_{ATP}/(mol_x\,h)$
P_{H2}	the partial pressure of hydrogen ($atm\,atm^{-1}$)
ΔG_{min}	the minimum energy of biochemical reactions (kJ/mol)
$C_{AH,e}$	extracellular concentrations of undissociated organic acid (mol/L)
$C_{AH,i}$	intracellular concentrations of undissociated organic acid (mol/L)
V_i	the production rate of metabolic acid (mol/(L h))

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