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RESEARCH PAPER

Evaluation of High Butanol/Acetone Ratios in ABE Fermentations with Cassava by Graph Theory and NADH Regeneration Analysis

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Abstract Higher butanol/acetone ratio is always desirable in ABE fermentation, and this ratio is closely associated with the complicated patterns of metabolic reactions and NADH generation rate. The patterns of acetate/butyrate formation and re-assimilation in multiple closed reaction loops, as well as NADH regeneration in ABE fermentation using different substrates varies. In this study, we evaluated butanol/acetone ratio in ABE fermentations utilizing cassava and corn based media by graph theory and NADH regeneration analysis. The theoretical calculations and experimental data revealed that a lower metabolic strength in butyrate loop and enhanced NADH generation rate were responsible for the achievement of higher butanol/acetone ratio when fermenting cassava based substrate. In traditional fermentations and extractive fermentations with oleyl alcohol/bio-diesel as the extractants when using cassava based substrate, butanol/acetone ratios reached 2.24, 2.84, and 2.19 with the increasing increments of 14.9, 61.4, and 6.8% respectively, while butanol productivities stayed at comparably high levels as compared with those of the fermentations when cultivating on corn based substrate.

Keywords: butanol, cassava, *Clostridium acetobutylicum*, graph theory, metabolic analysis, NADH generation

1. Introduction

As a clean/renewable fuel and a platform chemical, bio-butanol production *via* acetone-butanol-ethanol (ABE) fermentation has been recognized as a solution in dealing with problems of environmental pollution and diminishing fossil fuels [1]. Corn starch has been used as the major material for bio-butanol production by *Clostridium acetobutylicum* spp for over centuries. However, high cost of corn starch has been identified as a major factor affecting the economic viability of ABE fermentation [2]. Among the available starch substances for bio-butanol production, cassava is very attractive for its natures of cheapness, high productivity, and non-competition with food crops for arable land [3]. In ABE fermentation, solvent products of butanol, acetone, and ethanol are produced in a ratio of 6:3:1 (selective butanol/acetone ratio of about 2:1). Selectively increasing butanol yield or butanol/acetone ratio has always been a key issue in improving ABE fermentation. Researches have been successfully implemented aiming at achieving higher butanol/acetone ratios, including oxidative-reductive-potential control [4], utilization of mixed substrates [5], screening of hyper-butanol strains by metabolic engineering methods [6,7] and addition of electron carriers such as neutral red [8] and methyl viologen [9] into broth to inhibit hydrogenase in electron transport shuttle system to overproduce NADH. However, achievements of higher butanol/acetone ratios in above mentioned reports were at the expense of scarifying total solvents/butanol productivities or increasing purification loads.

One major problem in ABE fermentation is the severe growth inhibition from butanol. To overcome the problem, in-situ extractive fermentation was developed to relieve butanol inhibitory effect and to enhance the fermentation productivity [10]. Among various in-situ fermentation extractants for butanol, oleyl alcohol has been recognized as the best one, therefore, it is reasonable to use it as the

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protocol for investigating other features in ABE fermentation. In addition, bio-diesel selectively extracting certain amount of bio-butanol could be directly utilized as a kind of "properties improved" bio-diesel with higher cetane number (CN), which could significantly increase the commercial value of bio-diesel products while at the same time save distillation energy cost for at least more than 50% [11]. If butanol/acetone ratio could be up-regulated, then more butanol would be extracted into bio-diesel phase in ABE extractive fermentation with bio-diesel as the extractant and further quality improvement in the "properties improved" bio-diesel could be expected.

ABE fermentation is characterized with organic acids (butyrate and acetate) formation and re-assimilation in multiple closed reaction loops. Estimation of butanol/acetone ratio under special reaction loops and different conditions will lead to a better understanding of how the entire carbon metabolism is regulated. Directed signal flow diagram has been applied to simplify the metabolic networks [12,13]. Directed signal diagram was first proposed by Mason in 1956 [14], who represented the complicated electronic circuits with current directions and closed-loops by signal flow graph, called Mason Theory or graph theory. After that, a couple of its applications have been reported in the fields of analysis/estimation of carbon/energy metabolisms and recognition of physiological states in yeast and *E. coli* cultivations [12,13,15]. Graph theory treats the entire metabolic network as a model based on directed signal flow diagram, using substrates and end-metabolites as the inputs and outputs of the model respectively. The overall transfer function in between output and input could then be formulated by integrating all sub-transfer functions in between the intermediate nodes. In general, the transfer function from one intermediate node (A) to another one (B) could be represented by the stoichiometric coefficient of reaction A→B. On other hand, the generation rate/pattern analysis of co-factor such as the reductive power NADH is also very important in enhancing butanol/acetone ratio. In ABE fermentation metabolism, the central carbon flow branches at the node of Acetoacetyl-CoA. Acetone forms in one flow under the regulation of several coenzymes; while butanol forms in another flow by the catalysis of NADH and multiple NADH-dependent coenzymes. Acetone formation consumes neither NADH nor H_2; but butanol formation must consume certain amount of NADH and H_2. Intercellular NADH comes from two routes, EMP and the electron transport shuttle system. Addition of electron carrier such as neutral red or methyl viologen causes excess of proton and forces the proton to act with ferredoxin-NAD reductase to overproduce NADH, resulting in an enhanced butanol metabolic flux [8,9].

In our previous study, we demonstrated that timely and adequate addition of tiny amount of yeast extract (2.5 g/L-broth) could promote phase shift (acidogenic phase → solventogenic phase) by increasing transcriptional level of *ctfAB* to 16-fold when using cassava based medium, and butanol productivities in fermentations using cassava medium could be maintained at comparably high levels as compared with those of the fermentations using corn based medium [16]. In this study, we used graph theory to estimate butanol/acetone ratio and a simplified metabolic model to evaluate NADH generation rate for bio-butanol productions when utilizing cassava and corn based media, to show the nature of the enhanced butanol/acetone ratio when fermenting cassava based medium.

2. Materials and Methods

2.1. Microorganism

Clostridium acetobutylicum ATCC824 was maintained as spore suspension in 5% corn flour medium at 4°C. The methods of inoculation and pre-culture followed those described in our previous reports [11,16].

2.2. Medium preparation and *in-situ* fermentative extractants pre-treatment

The corn flour (raw starch content about 50% w/w) was obtained at local market and cassava powder (raw starch content about 65 ~ 70% w/w) was provided by Henan Tianguan Fuel Ethanol Co. Ltd., China. The media were pretreated by adding a tiny amount of α-amylase (8 U/g-corn or 8 U/g-cassava, heated in boiling water bath for 45 min) and then glucoamylase (120 U/g-corn or 120 U/g-cassava, heated at 62°C for 60 min). Subsequently, the viscosity-reduced media were autoclaved at 121°C for 15 min. Besides traditional ABE fermentation, extractive fermentations were also conducted using oleyl alcohol (Tokyo Kasei Co. Ltd., Japan) and bio-diesel (the chemically synthesized soybean one, provided by Huahong Biofuel Co., China) as the in-situ extractants. Oleyl alcohol and bio-diesel were either sterilized at 121°C for 20 min or directly used without sterilization, and then added into the fermentor. Concentrated yeast extract solution was sterilized at 115°C for 30 min, and it was pumped into the broth upon requirements [11].

2.3. Fermentation method and condition

Seed culture was carried out in 100 mL anaerobic fermentation bottles using corn flour as the substrate. The initial corn/cassava powders contents for traditional fermentations, extractive fermentations with bio-diesel and oleyl alcohol as the extractants, were 15 and 25%, respectively. The fermentations were conducted in a 7 L static fermentor

(Baoxing Bioengineering Co., China) equipped with pH and ORP (oxidative-reductive-potential) electrodes and a manual pressure adjustment unit. A temperature-controllable water bath (MP-10, Shanghai Permanent Science and Technology, Co., China) was used to circulate hot water into the coil pipes settled inside the fermentor to maintain broth temperature at 37°C. The fermentation medium loading volume ranged from 1.8 to 2.5 L, and the equivalent volume of oleyl alcohol or bio-diesel was added (for extractive fermentations) to ensure the volumetric ratio of oil/broth at 1:1. N_2 was firstly sparged into the extractant reservoir to remove the residual oxygen for 10 min. 10% (v/v) inoculum was then transferred into the fermentor and the broth was also sparged with N_2 for 10 min. In the extractive fermentations, the oxygen-free oleyl alcohol or bio-diesel were poured into the fermentor using a peristaltic pump after inoculation. The initial pressure inside the fermentor was controlled at about 0.02 MPa (N_2) to strictly maintain the anaerobic condition. The pressure gradually increased as fermentation started and self-generated gas began to evolve. The pressure was then controlled in a range of 0.030 ~ 0.055 MPa throughout fermentation. Agitation was occasionally exerted for a short time (5 min, 400 rpm) to promote butanol diffusion from aqueous phase into extractant phase.

2.4. Analytical methods

The measurements of concentrations of solvents, organic acids, glucose and starch were the same as those described in our previous reports [11,16]. After fermentations had proceeded for several hours, the total evolved gas amount was measured by collecting the gas in a graduated tube filled with water every hour. At 2 ~ 3 h intervals, the gas was firstly directed into two absorption bottles filled with 6 M NaOH solution and connected in-series to determine H_2 production amount within a period of 15 min, and then the total evolved gas amount in the remaining 45 min (measurement window length of 1 h) was quantified without passing the gas through the absorption bottles. In this way, H_2 and CO_2 release rates (r_{H2}, r_{CO2}), as well as H_2/CO_2 ratio during this measurement window could be calculated using Eq.1, with the assumption that H_2 and CO_2 were the only two components in the gas.

$$r_{H2} = A_{H2} \times 4 \qquad r_{CO2} = A_{GAS} \times 1.33 - A_{H2} \times 4$$

$$H_2/CO_2 = \frac{r_{H2}}{r_{CO2}} = \frac{A_{H2} \times 4}{A_{GAS} \times 1.33 - A_{H2} \times 4} \tag{1}$$

where A_{H2} and A_{GAS} were H_2 formation amount measured in the first 15 min and total gas evolved amount in the remaining 45 min, respectively. The units of r_{H2} and r_{CO2} were then converted into mol/L-broth/h using the ideal gas equation (25°C, 1 atm). r_{H2} or r_{CO2} was further smoothed by a polynomial using fermentation time as the independent variable, so that H_2 and CO_2 release rates at any arbitrary instant t ($r_{H2}(t)$, $r_{CO2}(t)$) could be determined. The acetone concentration was also smoothed by a polynomial using time as the independent variable, and its synthesis rate at instant t ($r_{ACE}(t)$) was then determined by differentiating the concentration with regards to time t.

2.5. ABE metabolic network

ABE metabolic network is shown in Fig. 1A according to the maps depicted in the literature [17]. The metabolic network was then reorganized or simplified as the directed signal flow diagram (Fig. 1B). Here, α_1, α_2, and α_3 represent the carbon flow distributions at acetyl-CoA (Ac-

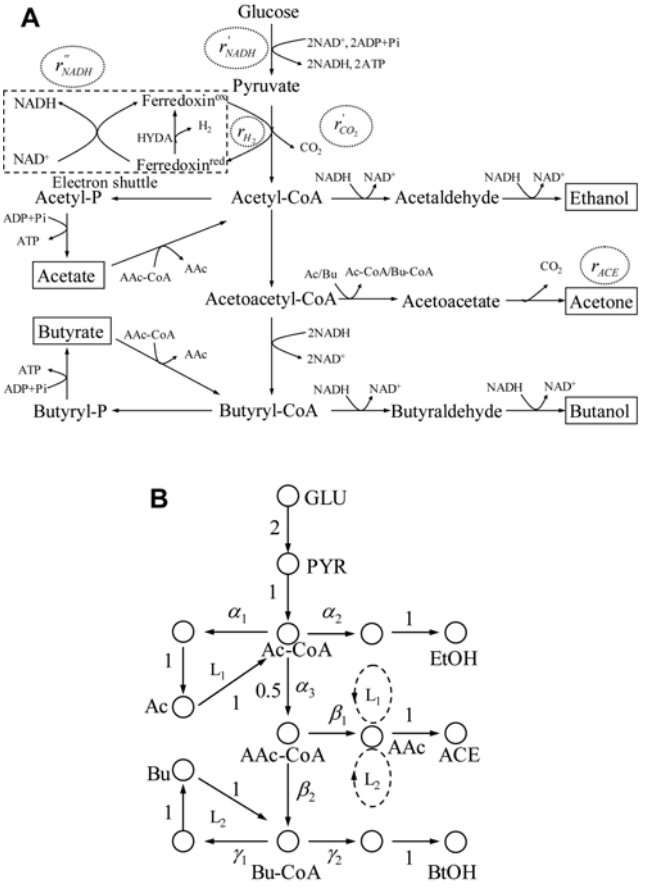
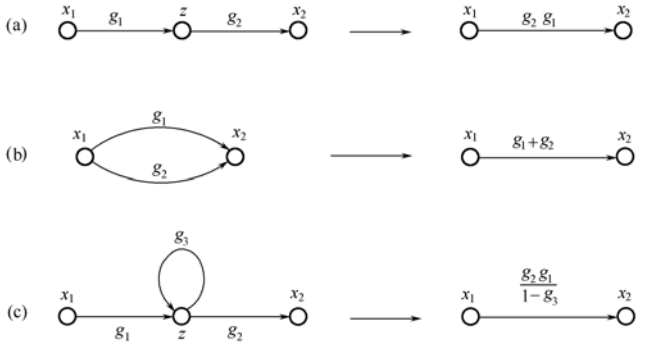


Fig. 1. The metabolic pathway and directed signal flow diagram of butanol biosynthesis by *Clostridium acetobutylicum* ATCC824. (A) The metabolic pathway. (B) The directed signal flow diagram reorganized from the metabolic pathway. HYDA: hydrogenase; GLU: glucose; PYR: pyruvate; Ac-CoA: acetyl-CoA; AAc-CoA: acetoacetyl-CoA; Bu-CoA: butyryl-CoA; BtOH: butanol; ACE: acetone; EtOH: ethanol; Ac: acetate; Bu: butyrate. r'_{NADH} and r''_{NADH}: rates of NADH generated in EMP route and electron transport shuttle system, respectively; r'_{CO2}: rate of CO_2 produced at Pyruvate → Acetyl-CoA reaction route; r_{ACE}: acetone formation rate; r_{H2}: H_2 release rate.

CoA) node for acetate loop (L_1), ethanol formation and central metabolic pass; β_1 and β_2 indicate carbon flow distributions at acetoacetyl-CoA (AAc-CoA) node for acetone and butanol synthesis paths; and γ_1 and γ_2 are the distributions for butyrate loop (L_2) and butanol formation at butyryl-CoA (Bu-CoA) node. "2" and "0.5" at Glucose (GLC) → Pyruvate (PYR) and Ac-CoA → AAc-CoA routes represent carbon stoichiometric coefficients of those reactions, while the rest stoichiometric coefficients are marked with "1" since those reactions are carbon number balanced.

2.6. Graph theory used for evaluation of butanol/acetone ratio

The graph theory (directed signal flow diagram) manipulates the signal transfer function from one node to another node with serial connection, parallel connection and intermediate closed-loop as follows, where g_1, g_2, and g_3 are the relevant sub-signal transfer functions between nodes or the sub-signal transfer function of a closed loop. If a signal transfer function from one node to another involving multiple loops is required for calculations, then the graph theory (Mason theory) must be adopted. Mason theory calculates the signal transfer function from node A to node i as follows [14].



$$T_{Ai}=\frac{\sum_k P_k\Delta_k}{\Delta} \qquad \Delta=1-\sum_l L_1+\sum_{l,m} L_1L_m-\sum_{l,m,n} L_1L_mL_n+\cdots \quad (2)$$

where Δ is called graph determinant, L_l is the transfer function of the l-th closed loop, and L_lL_m is the transfer functions multiplication of the l-th and m-th closed loops which are not in contact with each other. Similarly, $L_lL_mL_n$ is the transfer functions multiplication of the l-th, m-th and n-th closed loops which are not in touch with each other. P_k represents transfer function of the k-th forward path from point A to point i, and Δ_k is the surplus of Δ deleting all the transfer functions and their multiplication of the closed loops which are in touch with P_k.

Here, as an example, we demonstrated how to calculate the signal transfer functions from glucose node to butanol and acetone nodes as shown in the directed signal flow diagram of Fig. 1B. Under the assumption that acetate and butyrate loops (L_1 & L_2) simultaneously existed, then the signal transfer function from glucose node to butanol node (T_{GB}) could be calculated as follows.

$$T_{GB}=\frac{P_1\times\Delta_1}{\Delta}=\frac{(2\times0.5\times(1-\alpha_1-\alpha_2)\times\beta_2\times\gamma_2)\times1}{1-(L_1+L_2)+L_1L_2} \quad (3)$$

Here, since closed loops L_1 and L_2 were not in touch with each other, therefore, $\Delta=1-(L_1+L_2)+L_1L_2$. According to Fig. 1B, there was only one forward path (P_1) starting from glucose node (G) to butanol node (B). For butanol synthesis, the forward path P_1 was in touch with both L_1 and L_2, therefore $\Delta_1=1-(\cancel{L_1}+\cancel{L_2})+\cancel{L_1L_2}=1$. However, for acetone synthesis, the directed signal flow diagram treatment was somewhat peculiar, because Acetoacetyl-CoA (AAc-CoA) → Acetoacetate (AAc) reaction is associated with Acetate (Ac) → Acetyl-CoA (Ac-CoA, L_1) and Butyrate (Bu) → Butyryl-CoA (Bu-CoA, L_2) reactions as shown in Fig. 1A. Therefore, both L_1 and L_2 have to be supplemented at AAc node as shown in Fig. 1B (broken lines). In this way, signal transfer function from glucose node to acetone node (T_{GA}) could be summarized as follows, as closed loops L_1 and L_2 were in touch with each other at AAc node ($\Delta=1-(L_1+L_2)+\cancel{L_1L_2}=1-(L_1+L_2)$) and the only forward path P_2 from glucose node (G) to acetone node (A) was in touch with both L_1 and L_2 ($\Delta_2=1-(\cancel{L_1}+\cancel{L_2})+\cancel{L_1L_2}=1$).

$$T_{GA}=\frac{P_2\times\Delta_2}{\Delta}=\frac{(2\times0.5\times(1-\alpha_1-\alpha_2)\times\beta_1)\times1}{1-(L_1+L_2)} \quad (4)$$

When the signal transfer functions from glucose node to the ending nodes of butanol and acetone were determined, then the butanol/acetone ratio in weight base ($R_{BtOH/ACE}$) could be calculated using Eq. 5.

$$R_{BtOH/ACE}=\frac{T_{GB}\times M_{BtOH}}{T_{GA}\times M_{ACE}} \quad (5)$$

where M_{BtOH} and M_{ACE} were molecular weights of butanol and acetone, respectively. It must be addressed that the selective butanol yield (butanol content in the total solvent produced) is dominantly influenced by butanol/acetone ratio, ethanol was ignored in this case due to its small formation amount and low contribution to this yield.

2.7. Determination of NADH generation rate

Reductive power NADH also plays a very important role in regulating butanol/acetone ratio in ABE fermentation metabolism. As shown in Fig. 1A, NADH is generated in two paths, namely the EMP route and the electron transport shuttle system. In the EMP route, glucose is converted into Acetyl-CoA indicated by the following stoichiometric reaction.

$$\text{Glucose} \rightarrow 2\ \text{Acetyl-CoA} + 2\ \text{NADH} + 2\ CO_2 \quad (6)$$

Therefore, the NADH generation rate originated from EMP route (r'_{NADH}) equals to the evolution rate of CO_2 (r'_{CO2}) released in this path (Pyruvate → Acetyl-CoA (AC-CoA) + CO_2). On the other hand, the electron transport shuttle system produces another portion of NADH (r''_{NADH}) by the following co-reaction pairs of Pyruvate → Acetyl-CoA + CO_2 and ferredoxin^{ox} → ferredoxin^{red} to continuously operate the entire ABE fermentation system, where ferredoxin^{red} could be converted into ferredoxin^{ox} by the co-reactions of H_2 generation catalyzed by hydrogenase and NADH over-production as shown in Fig. 1A.

$$r'_{NADH} = r'_{CO2}$$
$$r_{AC-CoA} = r'_{CO_2} = r_{H_2} + r''_{NADH} \quad (7)$$

Another portion of CO_2 is released at the reaction route of Acetoacetyl-CoA → Acetone (ACE) + CO_2 in *Clostridia* metabolism. The CO_2 evolution rate in this route r''_{CO2} equals to acetone synthesis rate r_{ACE}, namely:

$$r''_{CO2} = r_{ACE}$$
$$r_{CO2} = r'_{CO2} + r''_{CO2} \quad (8)$$

Combing Eq. 7 and Eq. 8, the total NADH regeneration rate r_{NADH} could be formulated as follows.

$$r'_{NADH} = r'_{CO_2} = r_{CO_2} - r''_{CO2} = r_{CO2} - r_{ACE} \quad (9)$$

$$\begin{aligned} r_{NADH} &= r'_{NADH} + r''_{NADH} = r'_{CO2} + r'_{CO2} - r_{H2} = 2r'_{CO2} - r_{H_2} \\ &= 2(r_{CO2} - r''_{CO2}) - r_{H2} = 2(r_{CO_2} - r_{ACE}) - r_{H2} \\ &= 2r_{CO_2}\left(1 - \frac{r_{ACE}}{r_{CO_2}} - \frac{1}{2}\frac{r_{H_2}}{r_{CO_2}}\right) \end{aligned} \quad (10)$$

where r_{NADH}, r_{CO2}, r_{ACE} and r_{H2} represented for total NADH generation rate, total CO_2 evolution rate, acetone synthesis rate and H_2 evolution rate, respectively.

3. Results and Discussions

3.1. Enhanced butanol/acetone ratios in ABE fermentations using cassava based medium

In ABE fermentation, the first phase is acidogenic phase where organic acids forming pathways are activated, and acetate, butyrate, H_2 as well as CO_2 are produced. The second phase is solventogenic phase where the accumulated acids are re-assimilated in productions of acetone, butanol and ethanol. In our previous report, we found that the severe time delay in phase shift from acidogenic into solventogenic phase using cassava based medium could be eliminated by adding concentrated yeast extract solution into the broth (2.5 g/L-broth) at the instant when gas production tended to cease, leading to fermentation performance improvement. The activation of CoA-transferase encoded by *ctfAB* gene stimulated by yeast extract addition was responsible for this improvement. The transcriptional

Table 1. Fermentation performance under various operation modes with corn or cassava based media

Experimental results							
Batch #	Run time (h)	Final BtOH in aqueous phase (g/L)	Solvents concentrations (g/L-broth)		Butanol/acetone ratio (-)	BtOH productivity (g/L/h)	Gas production amount (L/L-broth)
			BtOH	ACE			
T1	65	11.63	11.63	5.97	1.95	0.179	23.26
T2	51	13.60	13.61	6.07	2.24	0.267	39.71
E-OA1	102	8.19	35.87 (27.68*)	20.37	1.76	0.352	86.13
E-OA2	100	8.62	41.92 (33.30*)	19.51	2.15	0.419	74.66
E-OA3	101	7.07	34.37 (27.30*)	12.12	2.84	0.340	77.74
E-OA4	99	6.60	29.81 (23.21*)	10.76	2.77	0.301	60.09
E-BD1	90	8.80	18.37 (9.57*)	8.95	2.05	0.204	32.87
E-BD2	90	8.37	19.47 (11.10*)	8.89	2.19	0.216	48.16
Simulation results (standard conditions)							
Corn based medium			L_1= 0.2, L_2= 0.2		2.23		
Cassava based medium			L_1= 0.2, L_2= 0.1		2.61		

T: Traditional fermentations; E-OA: Extractive fermentations with oleyl alcohol as the extractant; E-BD: Extractive fermentations with bio-diesel as the extractant.
T1: corn based medium; T2: cassava based medium with concentrated yeast extract solution addition at 21 h; E-OA1 and E-BD1: corn based medium; E-OA2: corn based medium but with concentrated yeast extract solution addition at 39 h; E-OA3 and E-OA4: cassava based medium with concentrated yeast extract solution addition at 39 h; E-BD2: cassava based medium with concentrated yeast extract solution addition at 28 h.
BtOH: butanol; ACE: acetone.
*: butanol concentrations in extractant phase.

level of *ctfAB* increased 16-fold after the concentrated yeast extract solution was supplemented [16]. In addition, over-productions of NADH and aspartic acid family which was favorable for butanol synthesis [18] after the concentrated yeast extract solution addition, also accounted for the recovery in solvents productivity. In this study, we cultivated *Clostridium acetobutylicum* ATCC824 on both corn and cassava (with yeast extract addition) based media to compare their fermentation performances. Both traditional and in-situ extractive fermentations (using oleyl alcohol and bio-diesel as the extractants) were conducted. The applications of extractive fermentations were to investigate many other features (besides butanol inhibition) in ABE fermentations since butanol inhibitory effect could be relieved and fermentation could continue for a much longer time in this case. The fermentation performance under various operation modes with corn and cassava based media was summarized in Table 1. Relatively higher butanol/acetone ratios were obtained in both traditional and extractive fermentations when fermenting cassava based medium. In traditional and extractive fermentations with oleyl alcohol/bio-diesel as the extractants, the ratios increased by 14.9, 61.4, and 6.8%, respectively, compared with those when fermenting corn based medium, while butanol productivities stayed at comparably high levels without much changes. It should be addressed that in the case of fermenting cassava based medium and using bio-diesel as the extractant, the higher butanol/actone ratio could increase butanol concentration in bio-diesel by 16%, and that is beneficial for further improvement in bio-diesel quality. To explore and interpret the reasons of the higher butanol/acetone ratios achieved when fermenting cassava based medium, theoretical calculations based on graph theory and NADH regeneration analysis were conducted.

3.2. Theoretical analysis of butanol/acetone ratios in bio-butanol productions fermenting cassava and corn by graph theory

In the theoretical calculation, the following parameters and equations were applied to simulate the "standard operation condition" when using corn-based medium as the substrate, where the metabolic strengths of acetate and butyrate loops (L_1 & L_2) were set at 0.2 (L_1=L_2=0.2). The parameters were so selected to ensure/allow carbon flow balances at the branch nodes and the overall system produce an approximate butanol/acetone/ethanol weight ratio ($W_{butanol}$:$W_{acetone}$:$W_{ethanol}$) of 6:3:1 as shown in Fig. 1A.

α_3:α_2=0.95:0.05, L_1=α_1, α_1+α_2+α_3=1;
β_2:β_1=0.7:0.3, β_1+β_2=1; L_2=γ_1, γ_1+γ_2=1.
→ $W_{butanol}$:$W_{acetone}$:$W_{ethanol}$=0.58:0.33:0.09 (11)

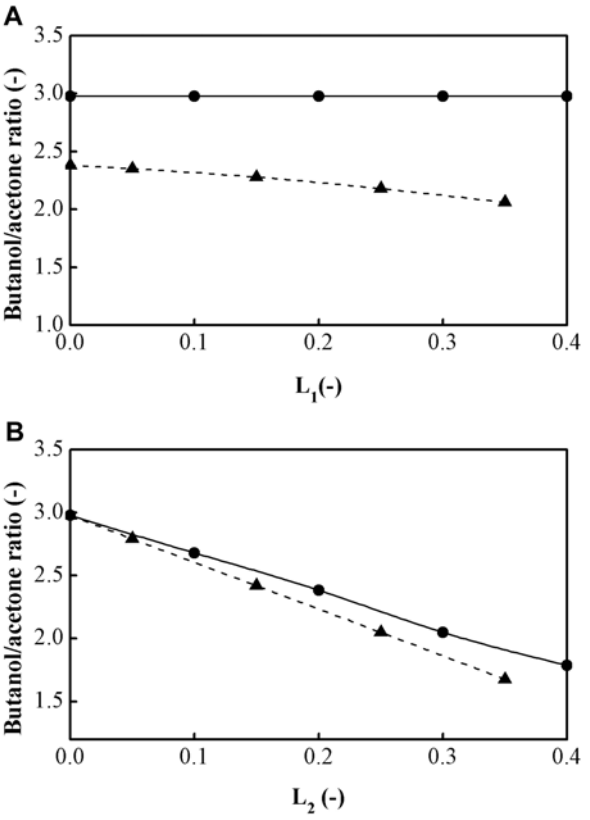


Fig. 2. The simulation and theoretical calculation results of butanol/acetone ratios using graph theory. The following parameters were used for calculations: α_3:α_2 = 0.95:0.05; β_2:β_1 = 0.7:0.3; L_1=α_1, α_1+α_2+α_3=1; L_2=γ_1, γ_1+γ_2=1. (A) Metabolic strength of L_1 loop (L_1) varied while that of L_2 (L_2) was fixed. ●: L_2 = 0.0; ▲: L_2 = 0.2. (B) Metabolic strength of L_2 loop (L_2) varied while that of L_1 (L_1) was fixed. ●: L_1 = 0.0; ▲: L_1 = 0.2.

The theoretical simulation results are depicted in Fig. 2. Theoretical calculation results revealed that the metabolic flux strength of butyrate formation and re-assimilation loop (butyrate loop, L_2) dominantly affected butanol/acetone ratio and a lower butyrate loop metabolic strength led to a higher butanol/acetone ratio. As shown in Fig. 2B, the ratio continuously decreased with the strength coefficient enlargement of L_2 loop (L_2) when L_1 loop strength (L_1) was fixed at 0.0 (butanol/acetone ratio decreased from 2.98 to 1.79, when metabolic strength of closed-loop L_2 increased from 0.0 to 0.4) or 0.2 (butanol/acetone ratio decreased from 2.98 to 1.68 when metabolic strength of closed-loop L_2 enhanced from 0.0 to 0.35). On the other hand, acetate loop (L_1) strength had less impact on the ratio. Butanol/acetone ratio stayed at the highest level of 2.98 consistently when L_2= 0.0, and decreased very slowly (butanol/acetone ratio decreased from 2.38 to 2.06 when metabolic strength of closed-loop L_1 changed from 0.0 to 0.35) when L_2 = 0.2 (Fig. 2A). The highest butanol/acetone ratio of 2.98 was

achieved if butyrate loop L_2 could be completely shut down ($L_2 = 0.0$). However, this ideal situation is actually not realistic or true, as the entire network has to be more or less related to acetate/butyrate formation/re-assimilation even in solventogenic phase.

Theoretical calculation results suggested that, in order to obtain a higher butanol/acetone ratio, the following measures have to be taken: (1) strict inactivation of butyrate loop (L_2). As shown in Table 1, the simulation results indicated that butanol/acetone ratio could be increased from 2.23 to 2.61 with an increasing increment of more than 17% if the metabolic strength of butyrate loop (L_2) was reduced 50% (from standard condition to optimal mode, L_2= 0.2→0.1 while L_1 was fixed at 0.2). (2) if butyrate loop (L_2) can not be effectively inactivated, then repression of acetate loop (L_1) strength coefficient is also desirable. These measures could be potentially realized by using hyper-butanol strains, adding electron carriers such as neutral red *etc*. to over-produce NADH, or fermenting other substrates with higher reductive power. However, the first choice might deteriorate the total solvents/butanol productivity or concentration [6,7,19]. Lee *et al*. [6] reported that batch cultivation of *adhE1*-complemented *C. acetobutylicum* M5 (pCAAD) strain resulted in non-acetone formation and increased molar butanol ratio (molar ratio of butanol to total solvents) from 0.61 to 0.89, as compared with those of the wild ATCC 824 strain. However, final butanol concentration stayed at 11.4 g/L (154.1 mM) and butanol productivity remained at a relatively low level of 0.170 g/L/h. Jiang *et al*. [7] reported when acetoacetate decarboxylase gene (*adc*) in *Clostridium acetobutylicum* EA 2018 was disrupted using TargeTron technology, butanol yield could increase to 80.1%, but total solvents productivity reduced from 0.398 to 0.194 g/L/h and butanol productivity reduced from 0.283 to 0.154 g/L/h. Sillers *et al*. [19] complemented the non-sporulating, non-solvent-producing *C. acetobutylicum* M5 strain (losing pSOL1 megaplasmid containing *aad* and acetone-formation genes) with alcohol/aldehyde dehydrogenase gene (*aad*) expressed from phosphotransbutyrylase promoter. The recombinant strain had no acetone accumulation and butanol yield could reach 87.3%. However, final solvents and butanol concentration were only 11.87 and 10.36 g/L.

The second choice, that is addition of electron carriers such as neutral red *etc*., would lead to heavy down-stream products purification loads since the electron carriers are basically pigments/dyes. As a result, the third choice seems to be more realistic. Butyrate closed-loop (L_2) neither produces nor consumes reductive power (NADH), but the major butanol synthesis route (Butyryl-CoA → Butanol) requires a large amount of NADH (Fig. 1A) consumption. As a result, utilization of the substrate with higher reductive power (NADH) is actually equivalent to butanol synthesis enhancement (γ_2 increase) and metabolic strength weakening of butyrate loop (L_2 decrease, $L_2 = \gamma_1$, $\gamma_1+\gamma_2=1$), which is favourable for increasing butanol/acetone ratio in turn.

3.3. ABE fermentation curves when using cassava and corn based media

ABE fermentation is characterized with acetate/butyrate formation and re-assimilation in multiple closed reaction loops during solventogenic phase and the strengths change of acetate/butyrate loops could influence production of butanol and acetone in this phase. Figs. 3A and 3B depicted the changing patterns of butyrate (L_2) and acetate (L_1) loops' metabolic strengths in traditional fermentations and extractive ones using corn and cassava based media, respectively. Both butyrate and acetate were the intermediate metabolites in solventogenic phase, and their concentrations actually represented the activated levels or strengths of L_1 and L_2 loops. A higher concentration implied a higher loop metabolic activity while a lower concentration suggested a lower loop activity or even a closed loop. Butyrate concentrations varied at relatively higher levels in the fermentations using corn based medium (Figs. 3A and 3B), while the concentrations stayed at relatively lower levels (about 50% of those of using corn based medium) in the fermentations using cassava based medium. These facts meant that metabolic activity of butyrate loop (L_2) when fermenting cassava based medium was significantly weakened. According to the theoretical analysis, a higher butanol/acetone ratio could be expected in this case. On the other hand, acetate concentrations stayed at equivalent levels using either cassava or corn based media, indicating that acetate loop metabolic strengths (L_1) were similar for both cases. ABE fermentation is a typical growth associated process, solvents production is closely related with cell growth. In addition, ABE fermentation is strictly anaerobic and ATP required for cell growth could not be generated by oxidative phosphorylation reaction. As shown in Fig. 1A, ATP could only be produced in acetate and butyrate loops (L_1 & L_2) so that acetate and butyrate formation and re-assimilation loops could not be completely shunt down or closed even in solventogenic phase. As a result, concentrations of acetate and butyrate could not reduce to zero level but stayed at an equilibrium level to maintain ATP generation. Butyrate closed-loop (L_2) neither produces nor consumes reductive power (NADH), but the major butanol synthesis route (Butyryl-CoA → Butanol) requires a large amount of NADH (Fig. 1A) consumption. As a result, utilization of the substrate with higher reductive power (NADH) such as cassava is actually equivalent to an enhanced butanol synthesis and weakened butyrate loop at Butyryl-CoA node. This is why the equilibrium butyrate concentration when using cassava-based medium is lower than that of

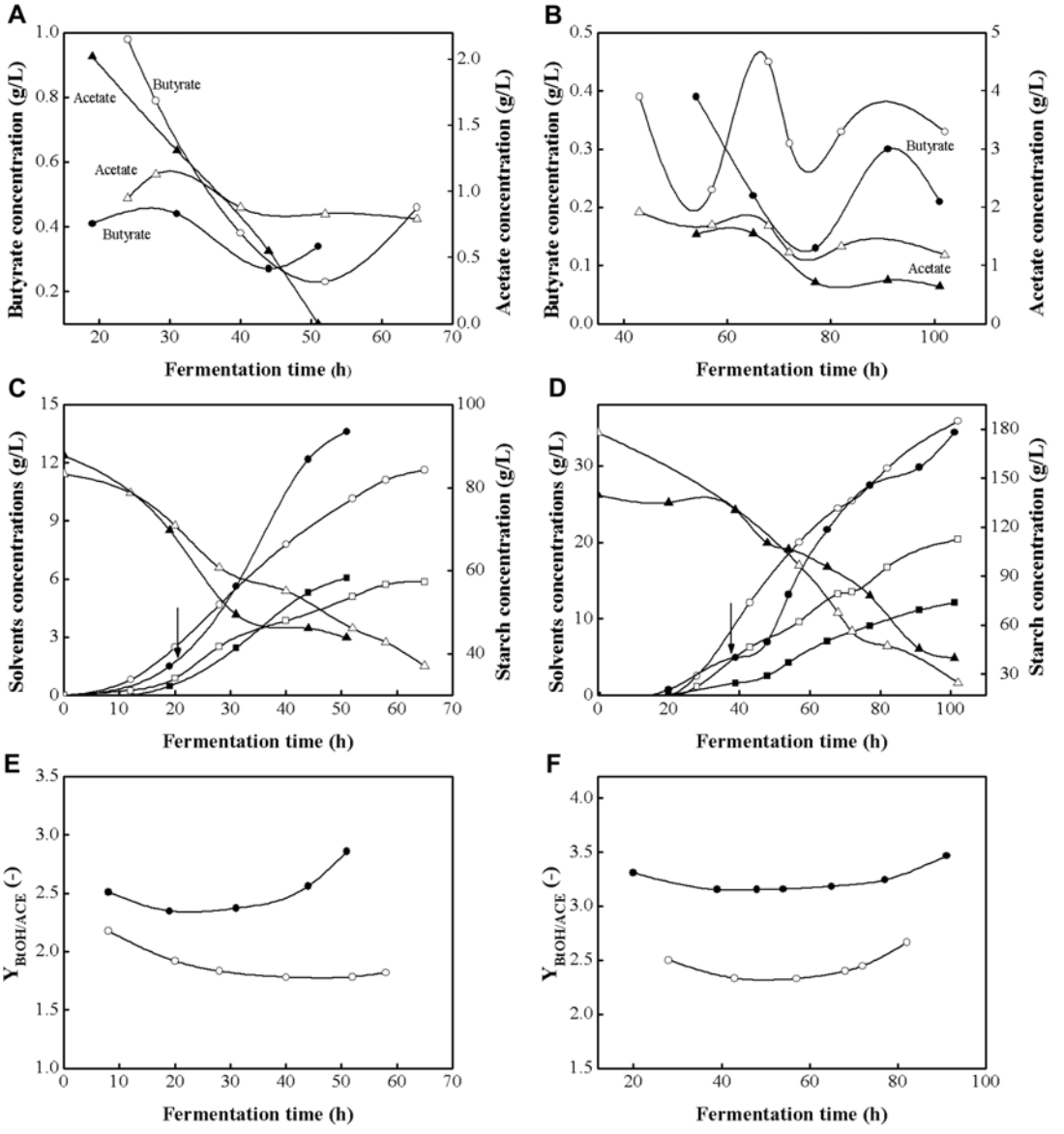


Fig. 3. Performance comparison of butanol fermentations using corn and cassava based media. (A, B) Organic acids concentrations. ●/○: butyrate concentrations; ▲/△: acetate concentrations. (C, D) Solvents and residual starch concentrations. ●/○: butanol concentrations; ■/□: acetone concentrations; ▲/△: starch concentrations. (E, F) The production ratios of butanol versus acetone ($Y_{BtOH/ACE}$). Solid symbols (●, ▲, ■): cassava based medium; open symbols (○, △, □): corn based medium. Arrow "→" represented the instant when concentrated yeast extract solution was added. (A, C, E) Results of traditional fermentation. (B, D, F) Results of extractive fermentation with oleyl alcohol as the extractant.

fermenting on corn-based substrate.

As shown in Table 1, the standard simulation condition with corn-based substrate and the "sub-optimal" simulation one with cassava-based substrate were set as follows, respectively: L_1= 0.2 & L_2 = 0.2 for corn medium; L_1 = 0.2 & L_2 = 0.1 for cassava medium. Under these simulated conditions, butanol/acetone ratio was expected to have an increasing increment of 17.1% from 2.23 to 2.61. In the experiments, the butanol/acetone ratios using cassava-based medium increased 14.9% in traditional fermentation and 61.4% in extractive fermentation with oleyl alcohol as the extractant, as compared with those of using corn-based medium (Table 1, Figs. 3C and 3D). The experimental butanol/acetone ratio's increase coincided with the simulation conclusion well, but the real increasing attitude in butanol/acetone ratio for extractive fermentation was much higher than that of the simulated one (61.4% versus 17.1%). In addition, as shown in Figs. 3E and 3F, in traditional fermentation and extractive fermentation with oleyl alcohol as the extractant, the production ratios of butanol versus

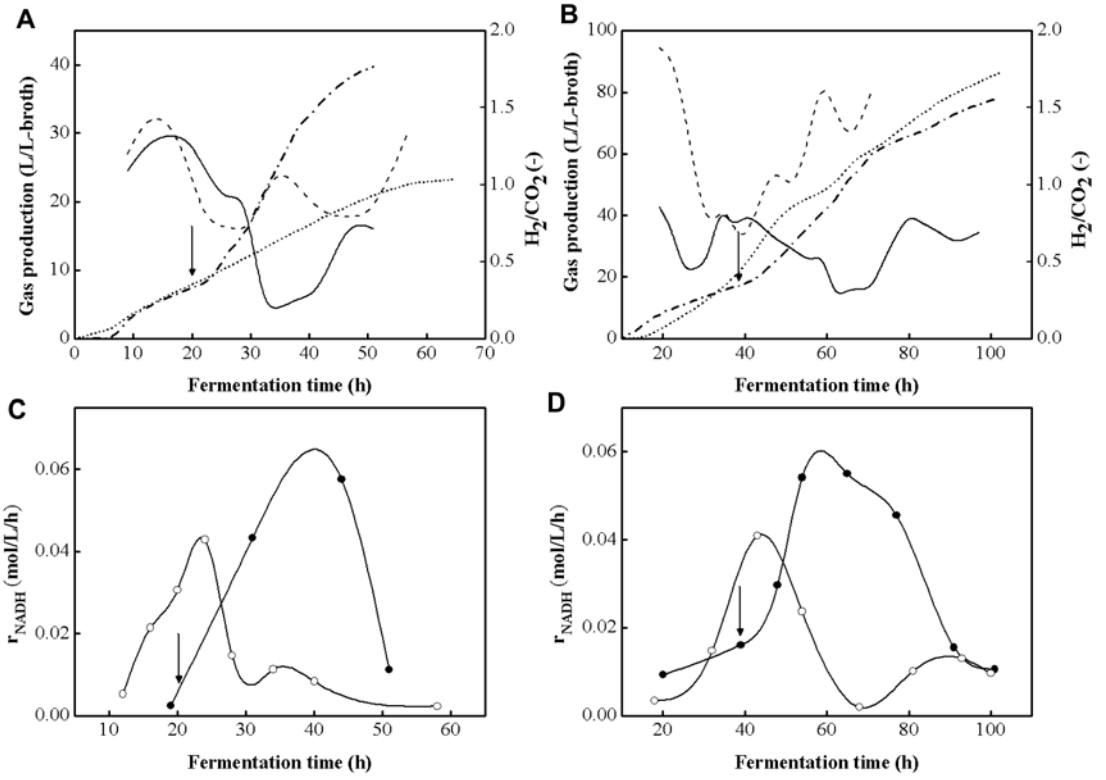


Fig. 4. Comparisons of gas production, H_2/CO_2 ratio, and NADH generation rate when using corn and cassava based media. (A, B) Gas production and H_2/CO_2 ratio. …: gas production amount using corn based medium; ---: gas production amount using cassava based medium; __: H_2/CO_2 ratio using cassava based medium; ---: H_2/CO_2 ratio using corn based medium. (C, D) NADH generation rate. ●: NADH generation rate using cassava based medium; ○: NADH generation rate using corn based medium. Arrow "→" represented the instant when concentrated yeast extract solution was added. (A, C) Results of traditional fermentation. (B, D) Results of extractive fermentation with oleyl alcohol as the extractant.

acetone ($Y_{BtOH/ACE}$) when fermenting cassava based medium were about 34.2 and 32.4% higher than those of using corn based medium. The higher values of $Y_{BtOH/ACE}$ were strongly associated with the overall butanol/acetone ratios when fermenting the cassava based medium.

3.4. Evaluation of high butanol/acetone ratios in bio-butanol productions fermenting cassava by NADH regeneration analysis

In *Clostridia* metabolism, H_2 is generated in the electron transport shuttle system by the catalysis of hydrogenase, and CO_2 is generated in Pyruvate → Acetyl-CoA and Acetoacetyl-CoA → Acetone routes. Figs. 4A and 4B depicted the changing patterns of H_2/CO_2 ratios in traditional fermentations and extractive fermentations with oleyl alcohol as the extractant, using corn and cassava based media respectively. Obviously, H_2/CO_2 ratios using cassava based medium were much lower than those of fermenting corn based medium in both fermentation modes. It is speculated that the less reductive power (NADH) might be one of the reasons limiting butanol synthesis. In the case of fermenting corn based medium, the electron transport shuttle system could not yield enough NADH to support butanol synthesis but released more H_2 by hydrogenase catalysis, leading to a higher H_2/CO_2 ratio but reduced butanol/acetone ratio.

The theoretical NADH generation rate calculated by Eq. (10) and using the experimental data of r_{CO2}, r_{ACE} and r_{H2} were plotted in Figs. 4C and 4D, for both of the traditional and extractive fermentations. Obviously, NADH generation rates when using cassava based medium were much higher than those of using corn based medium, and in the former case, NADH generation rates stayed at higher levels for much longer time. When NADH generation rates were maintained at higher levels, butanol accumulated quickly which could be observed in Figs. 3C and 3D. It must be addressed that, in the cases of fermenting cassava based substrate, rates of butanol synthesis and NADH generation rose up quickly and immediately after the yeast extract solution addition. This fact suggested that adding yeast extract solution into the cassava based medium indirectly stimulated NADH generation and butanol synthesis.

The nature of higher butanol/acetone ratio when fermenting cassava based substrate could also be interpreted by the

results reported in the literature. As shown in Fig. 1A, the existence of both acetate loop (L_1) and butyrate loop (L_2) with certain metabolic flux strength could produce extra intracellular ATP in solventogenic phase, which is beneficial for total solvents production, as the increased ATP promotes both cells growth and solvents production. However, enhanced ATP could only enhance cells growth and solvents production but could not alter butanol/acetone ratio. Girbal and Soucaille [20] reported that simultaneous existence of both higher intracellular ATP and butyrate concentrations could stimulate all the enzymatic activities involved with butanol and acetone formations, but the electron transport shuttle system was weakened leading to a less NADH production. The reduced NADH production led to a lower butanol/acetone ratio when using corn based medium. On the contrast, the ratio using cassava based medium appeared to be higher as metabolic strength of butyrate loop was inactivated.

There might be a concern whether the higher butanol/acetone ratio is really caused by fermenting cassava-based substrate since concentrated yeast extract (YE) solution was added during the fermentations. Therefore, extractive fermentation using corn-based medium as the substrate and oleyl alcohol as the extractant, with the concentrated YE solution (2.5 g/L-broth) addition at the same time (39 h) when implementing the same extractive fermentation on cassava-based substrate was carried out. The results indicated that YE addition in corn-based medium could not increase butanol/acetone ratio, and higher butanol/acetone ratio obtained in the case of fermenting cassava-based medium was completely due to the higher reductive power of the raw material instead of YE addition.

4. Conclusion

We estimated butanol/acetone ratio by graph theory and NADH theoretical calculation for bio-butanol productions utilizing both cassava and corn based media. The theoretical calculations and experimental data revealed that a lower metabolic strength in butyrate loop and enhanced NADH generation rate were responsible for the achievement of higher butanol/acetone ratio when fermenting cassava based substrate. In traditional fermentations and extractive fermentations with oleyl alcohol/bio-diesel as the extractants when using cassava based substrate, butanol/acetone ratios increased by 14.9, 61.4, and 6.8%, respectively.

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