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RESEARCH ARTICLE

High production of butyric acid by *Clostridium tyrobutyricum* mutant#

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Abstract The objective of this study was to improve the production of butyric acid by process optimization using the metabolically engineered mutant of *Clostridium tyrobutyricum* (PAK-Em). First, the free-cell fermentation at pH 6.0 produced butyric acid with concentration of 38.44 g/L and yield of 0.42 g/g. Second, the immobilized-cell fermentations using fibrous-bed bioreactor (FBB) were run at pHs of 5.0, 5.5, 6.0, 6.5 and 7.0 to optimize fermentation process and improve the butyric acid production. It was found that the highest titer of butyric acid, 63.02 g/L, was achieved at pH 6.5. Finally, the metabolic flux balance analysis was performed to investigate the carbon rebalance in *C. tyrobutyricum*. The results show both gene manipulation and fermentation pH change redistribute carbon between biomass, acetic acid and butyric acid. This study demonstrated that high butyric acid production could be obtained by integrating metabolic engineering and fermentation process optimization.

Keywords *Clostridium tyrobutyricum*, butyric acid production, fermentation, mutant, pH, flux balance analysis

1 Introduction

Butyric acid, a four-carbon short-chain fatty acid, has been widely used in chemical, food, and pharmaceutical industries. For example, butyric acid has been used as an additive in the manufacturing of plastic and fiber products to enhance their hydrophobicity, flexibility, and light and heat resistance [1]. Butyric esters are used as fruit-flavor fragrant reagent in beverages, food, and cosmetics [2]. Butyric acid has a great potential to treat colorectal cancer, hemoglobinopathies, and insulin resistance [3–5]. In addition, butyric acid can be used as a raw material to synthesize biofuel. During the last two decades, there has been an increasing interest to produce butyric acid from microbial fermentation because of the growing demand for bio-based natural foods, cosmetics, and pharmaceuticals [6].

The anaerobic Clostridial strains, such as *C. butyricum* [7], *C. tyrobutyricum* [8], and *C. thermobutyricum* [9], have been predominantly used in butyric acid production. *C. tyrobutyricum* ATCC 25755, a gram-positive, rod-shaped, and anaerobic bacterium, produces butyric acid as the main product from various carbohydrates, such as glucose and xylose [10]. The recent progresses of butyric acid production are summarized in Table 1 [11–21]. Compared with other strains, the wild type *C. tyrobutyricum* produced higher level of butyric acid in the fed-batch free-cell fermentation, 24.88–33.00 g/L *vs.* 6.30–19.40 g/L. Both metabolic engineering and fermentation process development have been applied to improve butyric acid production by *C. tyrobutyricum*. For example, one engineered mutant, *C. tyrobutyricum* PAK-Em, has been constructed by downregulating the acetate kinase gene (*ack*) involved in acetic acid formation pathway [22]. Although this mutant showed improved butyric acid production compared to wild type, the cell growth rate was reduced significantly. In addition, the metabolic flux analysis is an important tool to analyze the carbon redistribution caused by genetic engineering or fermentation process, but it has not been applied to investigate the mutant PAK-Em.

The major fermentation process parameters that affect butyric acid production include fermentation mode, agitation, temperature, pH, nutrient feeding strategy, etc [10]. A novel fibrous-bed bioreactor (FBB) has been developed to produce organic acids and biofuel in the immobilized-cell fermentations, which can significantly improve the productivity, yield, and final product con-

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Table 1 Recent progresses of butyric acid production from sugar

Strains	Fermentation mode	Sugar	Concentration /(g·L^{-1})	Ref.
C. populeti	Batch, free-cell	Glucose	6.30	[11]
C. butyricum ZJUCB	Batch, free-cell	Glucose	12.25	[12]
	Fed-batch, free-cell		16.74	
C. butyricum S21	Batch, free-cell	Lactose	18.60	[13]
C. beijerinckii	Batch, free-cell	Lactose	12.00	[14]
C. thermobutyricum	Fed-batch, free-cell	Glucose	19.40	[15]
C. tyrobutyricum JM1	Batch, free-cell	Glucose	13.76	[16]
C. tyrobutyricum, wild type	Fed-batch, free-cell	Glucose	24.88	[17]
	Continuous, free-cell	Glucose	33.00	[18]
C. tyrobutyricum, mutant	Fed-batch, immobilized-cell	Glucose	49.90	[19]
	Repeated fed-batch, immobilized-cell	Glucose	86.9	[20]
E. coli	Batch, free-cell	Glucose	10.00	[21]

centration by adapting and concentrating cell cultures with high tolerance against the toxicity of fermentation products [23–26]. In FBB fermentation, the butyric acid concentration has been improved by 2 to 3 folds because of the high cell density and the high butyric acid tolerance of the immobilized *C. tyrobutyricum* cell inside FBB [27,28]. As shown in Table 1, 49.9 g/L and 86.9 g/L of butyric acids have been produced by *C. tyrobutyricum* mutant from fed-batch FBB fermentation and repeated fed-batch FBB fermentation, respectively [19,20]. It has been reported that pH can significantly affect the butyric acid production [29], but its effect on PAK-Em has not been reported so far.

The main objective of this work was to achieve high butyric acid production by *C. tyrobutyricum* PAK-Em. The free-cell fermentation was performed to build the baseline of butyric acid production. The immobilized-cell fermentations using FBB were applied to improve the butyric acid production through pH optimization. The metabolic flux balance analysis was used to understand the carbon redistribution caused by metabolic engineering and fermentation parameter. This work demonstrated the great potential to produce high-level butyric acid using the metabolically engineered *C. tyrobutyricum* combined with fermentation optimization.

2 Materials and methods

2.1 Cultures and media

The wild type *C. tyrobutyricum* ATCC 25755 was maintained on the Reinforced Clostridial Medium (RCM; Difco, Kansa City, MO) plates in an anaerobic chamber (95% N_2, 5% H_2). The metabolically engineered mutant, PAK-Em with inactivated *ack* gene, was obtained from Yang's Lab [30]. The seed colony of PAK-Em mutant was maintained on RCM plates containing 40 μg/mL erythromycin (Em). Unless otherwise noted, all liquid cultures were grown at 37 °C in a modified Clostridial Growth Medium (CGM) with glucose as substrate following the protocol described previously [20].

2.2 Fermentation kinetic study

The kinetics of both free-cell and immobilized-cell fermentations of *C. tyrobutyricum* were carried out in a 3-L stirred-tank bioreactor. The detailed fermentation operation was described in previous studies [10,11]. To identify the optimal fermentation pH, the immobilized-cell fermentations using FBB were operated at different pH values, including 5.0, 5.5, 6.0, 6.5, and 7.0. The fermentations were performed in duplicate and the results were presented as the average with standard deviation. The fermentation samples were taken twice a day from the fermentor to analyze the cell growth and titrate the substrate and products (i.e., butyrate and acetate).

2.3 Analytical methods

The cell growth of *C. tyrobutyricum* was analyzed by measuring the OD_{600} of the cell suspension using a spectrophotometer (Biomate3; Thermo Fisher Scientific, Waltham, MA). A high performance liquid chromatography (HPLC, Shimadzu, Columbia, MD) system was used to analyze the concentration of glucose, butyrate, and acetate in the fermentation broth. The detailed methods can be found in our previous study [10].

2.4 Metabolic flux analysis

A constraint-based metabolic model of the central metabolism of *C. tyrobutyricum* [31] was constructed to perform flux balance analysis (FBA) of carbon and energy. The data collected from butyric acid fermentations in the

pH optimization study were used as the input of the FBA model. Total nine biochemical reactions were included into the FBA model and are displayed, alongside their functions, in Table 2.

3 Results and discussion

3.1 Kinetics of free-cell fermentation

Figure 1 shows the kinetics of the free-cell fermentations at pH 6.0 and 37 °C by the wild type (control) and mutant PAK-Em. The wild type entered the exponential phase at 7 h while the PAK-Em entered the exponential phase at 13 h post inoculation. Because the same operation in seed culture preparation and bioreactor inoculation were used, the longer lag phase in the fermentation by PAK-Em was caused by the gene manipulation and the resulted metabolic burden. Energy (ATP) is generated during the formation of acetate, so the downregulation of acetate pathway reduced energy efficiency and thus slowed down the cell growth that consumed energy. As shown in Table 3, PAK-Em had a lower growth rate (0.14 h^{-1}) than that of the wild type (0.21 h^{-1}). As discussed before [11], both the low ATP production and the metabolic burden caused by high production of butyrate reduced the cell growth rate. In addition, the yield of biomass in the fermentation was lower for PAK-Em (0.04 g/g) than for the wild type (0.06 g/g).

As shown in Fig. 1, both strains started to produce butyrate and acetate very slowly at the beginning of the free-cell fermentation, then the production of acids sped up from the log phase. The wild type stopped producing butyrate after 50 h while PAK-Em stopped after 80 h. The PAK-Em mutant generated butyrate at a final concentration of 38.44 g/L and acetate at 7.16 g/L, whereas the final concentrations of butyrate and acetate from the wild type fermentation were 19.24 g/L and 4.22 g/L, respectively. Butyrate titer produced by PAK-Em was much higher than that by the wild type. This carbon rebalance between acetate and butyrate has been also observed in previous study [11] by downregulation of *ack* gene in PAK-Em.

As shown in Table 3, the butyrate yield was increased from 0.34 g/g glucose by wild type to 0.42 g/g glucose by PAK-Em. The productivities of butyrate were 0.39 g/L·h^{-1} and 0.47 g/L·h^{-1}, selectivity of butyrate were 0.81 g/g and 0.74 g/g, butyrate/acetate ratios were 4.56 g/g and 5.36 g/g by the wild type and PAK-Em, respectively. It is clear that the PAK-Em mutant is a better butyrate producer than the wild type. Our butyrate baseline study was similar to the butyrate production in previous study [11].

The free-cell fermentation showed that the downregulation of acetate pathway increased the production of butyrate due to the carbon flux redistribution from C2 to C4 in the metabolically engineered strain. Because the acetate pathway is more efficient in energy production, the PAK-Em had a significantly reduced cell growth rate and biomass yield. These results indicated that the regulation of C2 pathway caused the global carbon redistribution and energy redistribution. Further evaluation of carbon and energy balance could reveal the whole picture of the metabolic flux shift in butyrate production, and will be discussed in the following flux balance analysis.

3.2 Effect of pH on butyric acid fermentation

To increase the butyrate production, the immobilized-cell fermentation by PAK-Em using fibrous-bed bioreactor (FBB) was performed. It was reported that the wild type *C. tyrobutyricum* could grow at pH 5.0−7.0 [29], so the

Table 2 Stoichiometric equations used in the FBA modeling of *C. tyrobutyricum*

Reaction No.	Biological function	Stoichiometric equation [a,b]
(1)	Biomass formation	2 Glucose + 1.75 NADH + 1.75 H^+ + 29.7 ATP → 3 $C_4H_{6.4}O_{1.72}N$ + 1.75 NAD^+ + 29.7 ADP + 29.7 Pi
(2)	Formation of pyruvate (glycolysis)	Glucose + 2 NAD^+ + 2 ADP + 2 Pi → 2 Pyruvate + 2 NADH + 2 H^+ + 2 ATP
(3)	Formation of AcCoA and CO_2	Pyruvate + CoA + Fd_{ox} → AcCoA + Fd_{red} + CO_2
(4)	Formation of H_2	Fd_{red} + 2 H^+ → H_2 + Fd_{ox}
(5)	Formation of NADH	Fd_{red} + NAD^+ ⇔ NADH + H^+ + Fd_{ox}
(6)	Formation of acetate	AcCoA + ADP + Pi ⇔ Acetate + CoA + ATP
(7)	Formation of BuCoA and water	2 AcCoA + 2 NADH + 2H^+ → BuCoA + 2 NAD^+ + CoA + H_2O
(8)	Formation of butyrate or acetate	BuCoA + Acetate ⇔ Butyrate + AcCoA
(9)	Formation of butyrate	BuCoA + ADP + Pi ⇔ Butyrate + CoA + ATP

a) Reversible reactions are indicated by '⇔' while irreversible reactions are indicated by ' → '
b) Pi = inorganic phosphate; Fd_{ox} = oxidized ferredoxin; Fd_{red} = reduced ferredoxin; BuCoA = butyryl-CoA; AcCoA = acetyl-CoA

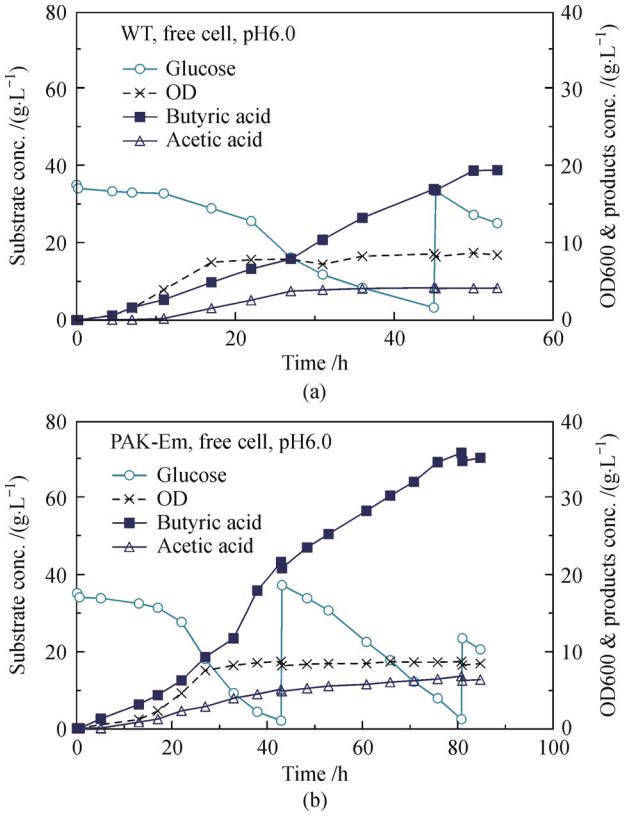

Fig. 1 Kinetics of free-cell fermentations by (a) *C. tyrobutyricum* ATCC 25755 wild type and (b) PAK-Em mutant at pH 6.0 and 37 °C. ○: Glucose, ■: Butyric acid, △: Acetic acid, ×: OD

immobilized-cell fermentations were run at pHs of 5.0, 5.5, 6.0, 6.5, and 7.0 to evaluate the effect of pH on the butyrate production by PAK-Em. The time-course data is presented in Fig. 2 and the production data of acids are summarized in Table 4.

As shown in Fig. 2, the glucose consumption rates in the immobilized-cell fermentation at all pHs were slow in the lag phase, increased greatly after cell growth entered log phase, and decreased in the late stationary phase. When strains stopped producing butyrate and acetate, there was no significant glucose consumption. The production of acids (butyrate and acetate) started at the beginning of the first batch and increased afterwards in the log phase. The production of acetate stopped in the early stationary phase and the butyrate concentration reached the maximum value at the end of fermentation. The fermentation timeline was significantly extended from 140−200 h at low pHs (5.0, 5.5 and 6.0) compared with that (280 h) at high fermentation pHs (6.5 and 7.0). Figure 2 also showed that the butyrate concentration was increased with the increase of fermentation pH.

As shown in Table 4, the immobilized-cell fermentation by PAK-Em produced the highest concentration of butyrate, i.e., 63.02 g/L, at pH 6.5. The butyrate/acetate ratio was also increased at high pHs (6.5 and 7.0). These results suggest that the uptake and conversion efficiency of glucose could be upregulated by higher pH and more carbon flux could be redistributed from C2 to C4.

The results in pH optimization demonstrated that the fermentation pH is an important process parameter to achieve high butyrate production. To optimize fermentation process, it is very important to understand how the pH correlates with the intracellular metabolic flux distribution. Moreover, the high concentration of butyrate produced at the optimized fermentation condition would greatly reduce the product extraction cost of butyrate, and thus make the biobutyrate production more competitive than the traditional butyrate production from petroleum.

3.3 Metabolic flux balance analysis

We constructed a flux balance analysis (FBA) model and analyzed the production data of biomass and acids in the immobilized-cell fermentations by wild type (fermentation data not shown) and PAK-Em at various pHs from 5.0 to 7.0. The balances of both carbon flux and energy flux were analyzed and presented in Fig. 3.

It was found that less carbon flux was distributed to the biomass production by PAK-Em than wild type at each pH, indicating that more carbon fluxed to the pyruvate in the PAK-Em. The node of acetyl-CoA and butyryl-CoA,

Table 3 Comparison of wild type (control) with PAK-Em in free-cell fermentation products [a,b,c)]

Products		Wild type (control)	PAK-Em
Cell growth	Growth rate μ /h^{-1}	0.21±0.01	0.14±0.01
	Biomass yield /(g·g^{-1})	0.06±0.01	0.04±0.01
Butyric acid	Concentration /(g·L^{-1})	19.24±0.05	38.44±0.03
	Yield /(g·g^{-1})	0.34±0.02	0.42±0.01
Acetic acid	Concentration /(g·L^{-1})	4.22±0.002	7.16±0.002
	Yield /(g·g^{-1})	0.07±0.001	0.07±0.01
C4/C2	B/A ratio /(g·g^{-1})	4.56±0.85	5.36±0.61

a)These free-cell fermentations were performed at pH 6.0
b)The data were presented as the average of duplicated fermentations with standard deviation
c)The biomass yield was calculated using 1 OD_{600} = 0.38 g/L [10]

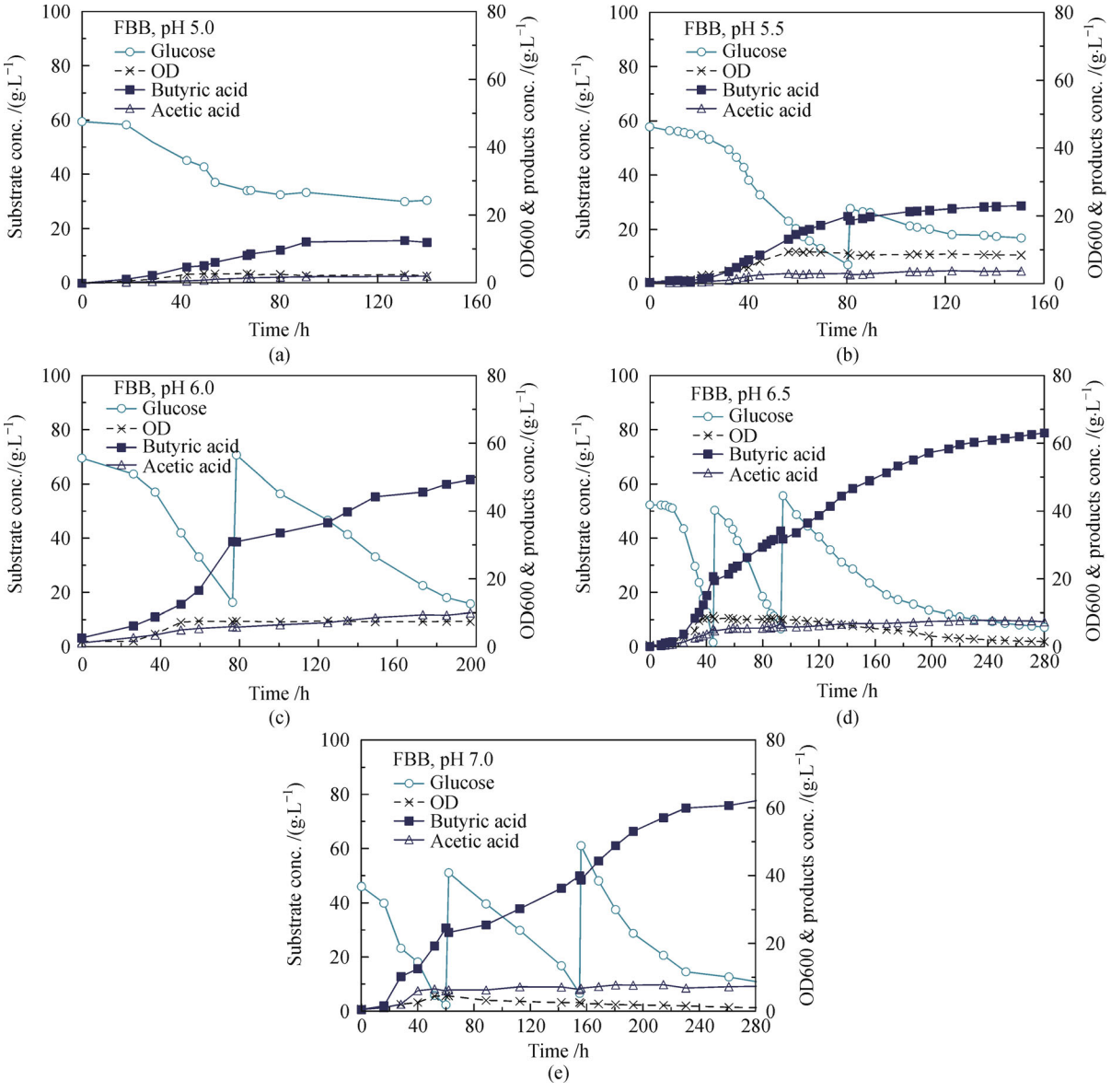

Fig. 2 Kinetics of immobilized-cell fermentations by PAK-Em at different pHs under 37 °C

Table 4 Effect of pH on acid production in immobilized-cell fermentations by PAK-Em

Products		pH				
		5.0	5.5	6.0	6.5	7.0
Butyrate	Conc. /(g·L^{-1})	14.79±0.99	23.18±0.78	50.11±2.42	63.02±1.54	61.01±0.78
	Yield /(g·g^{-1})	0.37±0.03	0.38±0.01	0.45±0.02	0.45±0.01	0.42±0.01
Acetate	Conc. /(g·L^{-1})	2.11±0.02	3.13±0.02	7.03±0.01	7.26±0.03	7.09±0.02
	Yield /(g·g^{-1})	0.03±0.004	0.03±0.006	0.08±0.01	0.05±0.01	0.04±0.01
C4/C2	Ratio /(g·g^{-1})	6.53	6.77	7.12	8.60	8.60

which are the key metabolites related to the metabolic flux from the C2 to C4, was influenced by the downregulation of acetate pathway and the culture pH levels. Compared to wild type, the PAK-Em directed a lower metabolic flux to acetyl-CoA (< 0.2 mol/mol-glucose) and a higher flux to the butyryl-CoA (> 0.8 mol/mol-glucose). As fermentation pH increased, more butyryl-CoA (from 0.83 to 0.91 mol/mol-glucose) was produced in the PAK-Em, which

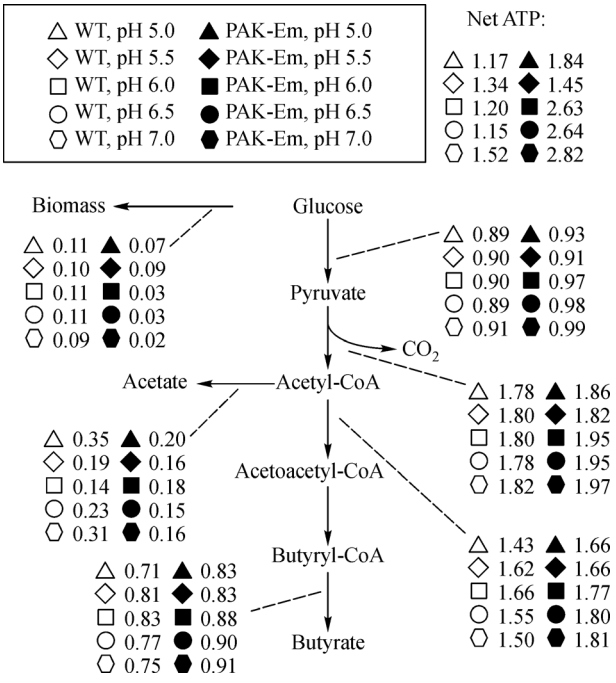

Fig. 3 *C. tyrobutyricum* metabolic flux distribution on a basis of 1 mole glucose consumed by wild type and PAK-Em at 37 °C and pH 5.0−7.0

was consistent with the higher concentration of butyrate.

In addition to providing the information of carbon flux distribution, FBA allowed for the calculation of net ATP accumulation, which is defined as the amount of ATP produced by glycolysis, acetate biosynthesis, and butyrate biosynthesis minus the ATP required for cell growth, by each strain at various pHs. The net ATP produced by the PAK-Em at pH 6.0−7.0 was significantly greater than that produced by the wild type in the same range of pH. Furthermore, the net ATP produced by both strains appeared to be largely independent of fermentation pH, excepting the jump in net ATP production by PAK-Em from 1.45 mol-ATP/mol-glucose at pH 5.5 to 2.63 mol-ATP/mol-glucose at pH 6.0. The increase of net ATP production observed in PAK-Em can be attributed to a redirection of carbon flux from biomass formation to acid synthesis. The decrease of carbon flux to biomass formation not only decreased the amount of ATP consumption by PAK-Em, it also increased the carbon flux participating in glycolysis that produced ATP, as well as increased the carbon flux to acetate and butyrate biosynthesis that produced ATP.

4 Conclusions

Taken together, the *C. tyrobutyricum* mutant PAK-Em obtained from integrational mutagenesis to selectively downregulate acetate pathway was used to produce high level of butyric acid with fermentation parameter optimization. This study demonstrated the feasibility and advantage of combining genetic engineering techniques with process optimization (i.e., environmental adaptation in FBB and pH optimization). The butyrate production was improved significantly to 63.02 g/L in the immobilized-cell fermentation at pH 6.5 by the PAK-Em. The high butyric acid concentration could reduce the production cost of bio-based butyric acid and allow its bioproduction to compete more favorably in the marketplace.

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