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REVIEW ARTICLE

High butanol production by regulating carbon, redox and energy in Clostridia#

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Abstract Butanol is a promising biofuel with high energy intensity and can be used as gasoline substitute. It can be produced as a sustainable energy by microorganisms (such as Clostridia) from low-value biomass. However, the low productivity, yield and selectivity in butanol fermentation are still big challenges due to the lack of an efficient butanol-producing host strain. In this article, we systematically review the host cell engineering of Clostridia, focusing on (1) various strategies to rebalance metabolic flux to achieve a high butanol production by regulating the metabolism of carbon, redox or energy, (2) the challenges in pathway manipulation, and (3) the application of proteomics technology to understand the intracellular metabolism. In addition, the process engineering is also briefly described. The objective of this review is to summarize the previous research achievements in the metabolic engineering of *Clostridium* and provide guidance for future novel strain construction to effectively produce butanol.

Keywords Clostridia, butanol, biofuel, metabolism, carbon, redox, energy

1 Introduction

Butanol is a representative of advanced biofuels, which is notable for its high energy density, low volatility, low hygroscopicity, and low corrosivity. Butanol shows an excellent compatibility with the current gasoline infrastructure as a fuel or a fuel additive. Butanol also plays an important role, such as solvent and precursor of plastic, in the chemical industry. In addition, butanol can be converted to acrylate, methacrylate esters, glycol ethers, and butyl acetate [1].

Butanol has been produced by various Clostridia with the development of fermentation technology [2]. For example, *Clostridium acetobutylicum* was firstly classified for the conversion of starch to acetone, butanol and ethanol in a ratio of 3 : 6 : 1 [3]. Strains that naturally produce butanol like *C. beijerinckii*, *C. ljungdahlii*, and *C. ragsdalei* have also been characterized and evaluated [4]. Although ABE fermentation has garnered continuous interest in recent years, it was difficult to reach high butanol production by engineering *C. acetobutylicum* because it is hard to manipulate the complicated metabolic pathway involved in acidogenesis, solventogenesis, and sporulation [5].

The acidogenic Clostridial strain, such as *C. tyrobutyricum* that produces acetate and butyrate as major products, has shown the feasibility to produce a high level of butanol after metabolic engineering [6]. The features of the relatively simple metabolic pathway, high butanol tolerance, and capability to metabolize low-value feedstock made *C. tyrobutyricum* a promising host microorganism in butanol production. Despite all these advantages, the lack of a full understanding of the metabolic pathway regulating mechanism hampered the development of novel mutant strains.

In this article, we summarized the progress achieved in butanol fermentation by Clostridia, especially the construction of metabolically engineered strain of *C. acetobutylicum* and *C. tyrobutyricum*. Various strategies to rebalance the flux of carbon, redox or energy were reviewed and discussed. The challenges in the manipulation of acid formation pathways were described. The application of proteomics technology to understand the intracellular metabolism was also presented. In addition to metabolic cell engineering, the process engineering in butanol production was also discussed.

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2 Carbon metabolism

As shown in the metabolic pathway of butanol production in Fig. 1, glucose is converted to pyruvate through the EMP pathway, and several products (acids and solvents) are produced subsequently. Pyruvate is catalyzed to lactate via Ldh, while acetyl-CoA is generated along with CO_2 via Pfor. The Pta and Ack are responsible for acetate production from acetyl-CoA, and Ptb and Buk catalyze the formation of butyrate from butyryl-CoA. In the wild type of acidogenic *C. tyrobutyricum*, no solvent (i.e., acetone, ethanol and butanol) is produced, but homogenous butanol dehydrogenase (*bdh*) gene exists.

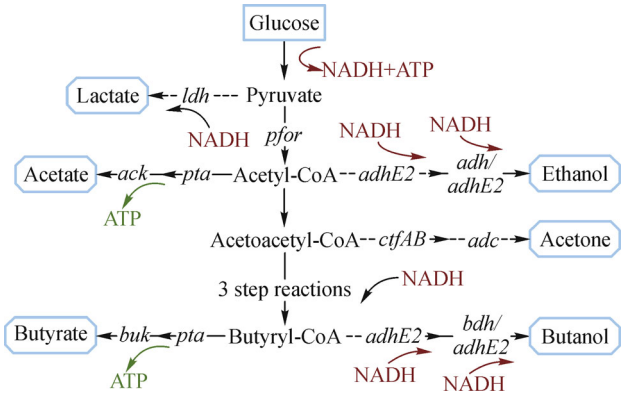

Fig. 1 Metabolic pathway of butanol production in Clostridia. Key enzymes and genes in the pathway: lactate dehydrogenase (*ldh*); pyruvate-ferredoxin oxidorecuctase (*pfor*); phosphotransacetylase (*pta*); acetate kinase (*ack*); phosphotransbutyrylase (*ptb*); butyrate kinase (*buk*); CoA transferase A and B (*ctfAB*); alcohol dehydrogenase (*adh*); butanol dehydrogenase (*bdh*); aldehyde-alcohol dehydrogenase (*adhE2* or *aad*). Dotted line indicates the pathway that does not naturally exist in the acidogenic strain such as *C. tyrobutyricum*

2.1 Manipulation of branched and central pathway

To improve butanol production, the genes involved in the acid producing pathways, such as *pta*, *ack*, *ptb* and *buk*, have been targeted. For example, the inactivation of the *buk* gene in *C. acetobutylicum* led to 42% of increase of butanol concentration [7], but the combination of *buk* inactivation and *aad* overexpression did not further improve the butanol production. Another study showed that the inactivation of *pta* and *buk* benefited the production of butanol with yield of 0.36 mol/mol glucose [8]. As expected, the carbon was redistributed from the downregulated acetate and butyrate pathways to the butanol pathway in a *pta-buk* deficient Clostridial strain. Meanwhile, the knockout of both *pta* and *buk* genes as well as the overexpression of *adhE* gene generated a strain with 60% increase of butanol titer and 154% increase of butanol yield.

In addition to the end-product generation pathways (i.e., branched pathways), the regulation of central pathway from acetyl-CoA to butyryl-CoA is a powerful method to increase butanol production. For example, the activity of thiolase (Thl) significantly affects the production of acetone, butyrate, and butanol. Hence the inactivation of *thl* could reduce or even block the formation of downstream products. Interestingly, the *thl* deleted mutant produced small amount of butanol but no butyrate, and the CoA transferase was not operational [9]. No butyrate was detected in *C. butyricum* when its *hbd* gene was inactivated, indicating that the alternative enzyme does not exist [10].

The metabolic engineering of Clostridia by combining the downregulation of the byproduct formation pathways and the upregulation of the central metabolic pathway to butyryl-CoA could improve butanol production [11]. However, unexpected results were observed in the butanol fermentation by the engineered strain. The root cause is that the strategy of metabolic engineering was not rationally designed and the interaction between different pathways was not fully understood. Therefore, the gene manipulation in Clostridia could cause the undesired output and the effort of metabolic engineering was traded off. For example, the CoA transferase in acetone pathway was downregulated by employing *ctfB* asRNA, but the butanol yield was decreased [12]. The further characterization indicated that the *ctfB* gene is located on the tricistronic operon (*aad-ctfA-ctfB*) and these genes reside on the same mRNA transcript. Therefore, the downregulation of *ctfB* with asRNA led to the decreased butanol due to the downregulation of the *aad* gene that was involved in the formation of butanol and ethanol [13]. Another study demonstrated that more butanol was produced in *C. tyrobutyricum* with the overexpression of *ctfAB* [14].

In *C. acetobutylicum*, it is hard to eliminate ethanol but keep butanol production because the Aad enzyme catalyzes the generation of both ethanol and butanol. Different from *C. acetobutylicum*, the *C. tyrobutyricum* contains different alcohol dehydrogenase genes in the ethanol and butanol formation pathways. The alcohol dehydrogenase (*adh*) gene is missed but the butanol dehydrogenase (*bdh*) gene exists in *C. tyrobutyricum* [15], thus introducing heterologous alcohol/aldehyde dehydrogenase (*ahdE2*) gene to *C. tyrobutyricum* could complete the butanol pathway but not the ethanol pathway. The results from both butanol fermentation and proteomics study confirmed this finding. This metabolic engineering in *C. tyrobutyricum* demonstrated that the synthesis of heterologous *adhE2* gene enabled the production of butanol [16].

2.2 Challenges in eliminating the formation of acids

The inactivation of *buk*, *pta*, and *ack* genes in *C. acetobutylicum* did not completely delete the production

of acids. For instance, the downregulation of acetate pathway in *C. acetobutylicum* by Cooksley et al. [9] and Kuit et al. [17] increased the solvent production but a significant of acetate production was still observed. It was postulated that the existence of acetate: butyrate-CoA CoA transferase provided an alternative pathway for acetate formation [18]. A butanol formation model was described in Fig. 2. The pathway generating butanol directly from acetyl-CoA through butyryl-CoA was called "hot channel", whereas the pathway via the re-assimilation of acetate and butyrate to generate butanol was named as "cold channel". Previous study showed that deleting the cold channel decreased the acetate and butyrate production but the yield of butanol was rather low even though butanol was produced only through the hot channel [8]. In addition, the mass balance analysis demonstrated that the hot channel had a more important role than the cold channel. Obviously reducing the cold channel flux cannot improve butanol production unless all the acid production pathways are blocked simultaneously.

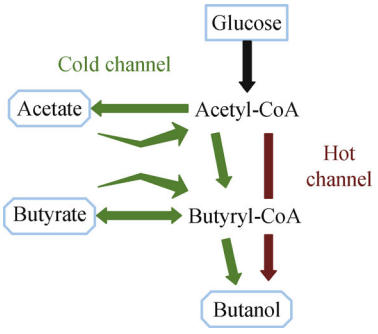

Fig. 2 Butanol formation routes: directly through hot channel and through cold channel via acid re-assimiation

Multiple studies were performed to manipulate the acetate pathway or butyrate pathway by the deletion or inactivation of single gene, but various results were reported. For example, Lehmann et al. obtained a *ptb* mutant that did not produce butyrate [19], while Jang et al. reported that the mutant that targeted the same insertion site had comparatively high enzyme activity of Ptb [8]. Moreover, Cooksley et al. obtained a putative *ptb* mutant after several attempts including three ClosTron constructs [9], but the mutant had inactivated *thl* gene rather than *ptb* gene. An *ack* knock-out mutant of *C. tyrobutyricum* reduced acetate kinase activity by ~50% but produced almost the same amount of acetate as the wild type [15]. Likewise the activities of Ptb and Buk decreased by 76% and 42% respectively by inactivating the corresponding genes through homologous recombination, while butyric acid production was not significantly reduced probably due to the compensation of isoenzymes. At the same time, both the activities of Pta and Ack and the production of acetic acid and hydrogen increased.

Gene knockout has been widely used in the down-regulation of acid formation pathways in Clostridia. The homologous recombination has also been widely used and a certain number of mutants were reported prior to 2007 [20]. A promising tool using the ClosTron Group II intron directed mutagenesis was developed by Heap et al. [21]. It had been successfully applied in several species, including *C. acetobutylicum*, *Clostridium difficile*, *Clostridium botulinum*, *C. beijerinckii*, *Clostridium sporogenes*, *Clostridium perfringens*, *Clostridium sordellii* [22], and recently *C. butyricum* [10]. However, the ClosTron technique could not provide an absolutely correct insertion in a targeted gene under all conditions due to the gene variability and the limitation of specific site recognition [9].

2.3 Fundamental understanding by systems biology

The regulation of the metabolism in *C. acetobutylicum* involves multiple phases, i.e., acidogenesis, solventogenesis, and sporulation [23]. It was reported that the fermentation pH, glucose uptake, and acid level during the transition from acidogenesis to solventogenesis could affect the production of solvents [24]. In *C. tyrobutyricum*, the metabolic flux to butyrate could be directly converted to butanol by synthesizing a butanol pathway and the transition from acidogenesis to solventogenesis was not observed. It is clear that the mechanisms of the solvent production by *C. acetobutylicum* and *C. tyrobutyricum* are different. Systems biology is a powerful tool to develop an in-depth understanding of the intracellular metabolism of butanol production.

The proteomic analysis was applied to investigate the expression of the genes involved in the central pathway (Fig. 3) in the solventogenic and non-solventogenic *C. acetobutylicum* strains, such as WT (wild type) and M5 mutant strain with the deletion of megaplasmid pSOL1 and

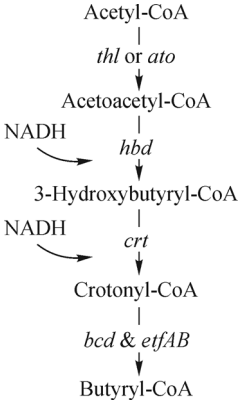

Fig. 3 Central pathway of *Clostridium* butanol formation. Enzymes and genes: acetyl-CoA-acetyl transferase (*ato*); thiolase (*thl*); beta-hydroxybutyryl-CoA dehydrogenase (*hbd*); crotonase (*crt*); butyryl-CoA dehydrogenase (*bcd*); electron transferring flavoprotein (*etf*)

the loss of solvent production [25]. In solventogenic strain, the expression of Adh1 and CtfB increased significantly after the phase transition while the expression of Buk decreased. In non-solventogenic strain, no Adh1 and CtfB activity was observed although Buk expression increased post phase transition. The carbon flux from acetyl-CoA to butyryl-CoA in the acidogenic strain was higher than that in solventogenic strain. A similar result was also reported in the comparative proteomics study of a high butanol producing mutant (*ack* knockout and *adhE2* overexpression) and high butyrate producing mutant (*ack* knockout) of *C. tyrobutyricum*, while the activities of Bcd and EtfAB were slightly lower in these mutants than in wild type [15]. In addition, the protein expression results indicated that the Bcd was more likely to be a bottleneck of butanol fermentation.

Different from *C. acetobutylicum*, *C. tyrobutyricum* begins the butanol production at the exponential phase without apparent acidogenic phase; it has a mixed phase producing solvents and acids simultaneously. A mutant with *ack* inactivation and *adhE2* overexpression has showed significantly increased activities of Thl, Ato, Hdb, and Crt [15]. In the comparative proteomics of *C. tyrobutyricum*, the mutants with high butanol productivity had lower expression level of glycolytic enzymes than wild type [15], which was similar to the protein expression result in *C. thermocellum* treated with ethanol stress [26]. It was found that the increased production of C4 products (butanol and butyrate) could be driven by the carbon redirection from acetyl-CoA to butyryl-CoA.

3 Redox metabolism

Redox is an indicator of the electron transfer involved in intracellular metabolism. It was found that the NADH/NAD^+ ratio was very important to redistribute metabolic flux [27]. Because 4 moles of NADH are consumed for the production of 1 mole of butanol, it would be expected that the NADH could limit the formation of butanol in the mutant with rebalanced carbon [28]. This hypothesis was confirmed by the following study results: 1) the reduction of NADH level occurred in the phase transition of *C. acetobutylicum* [29], 2) the redox engineering in *E. coli* produced high level of ethanol by increasing NADH availability [30], and 3) the decrease of NADH level in *Saccharomyces cerevisiae* decreased the production of glycerol [31]. All these previous studies indicated that redox plays an important role in solvent production. Redox can be improved by upregulating NADH formation pathway or downregulating the NADH consumption pathway.

3.1 Cellular regulators of redox balance

It was reported that the ratio of intracellular NADH/NAD^+ varied at different fermentation pHs, indicating that pH is essential for butanol production in Clostridia. The culture pH can affect the reducing power, enzymes activity, duration of lag phase, and the interaction of metabolic pathways [32]. For example, the expression of Bdh in *C. beijerinckii* was affected by pH [33], and the pH optimization could redirect the metabolic flux toward more hydrogen production [34].

The hydrogen production could correlate with NADH metabolism in Clostridia. Three metabolic pathways that regulate the NADH/electron flow have been investigated: (1) NADH-ferredoxin reductase and hydrogenase [35], (2) Bcd/EtfAB complex and hydrogenase involved in the reaction of "2 NADH + 1 oxidized ferredoxin + 1 crotonyl-CoA = 2 NAD^+ + 1 ferredoxin + 1 butyryl-CoA" [36], and (3) bifurcating hydrogenase oxidizing NADH and ferredoxin simultaneously [10].

Multiple enzymes in the central metabolic pathway of butanol production can regulate the metabolism of NADH. For example, the Bcd/Etf complex consumed the reducing power in Clostridia [37]. The NADH-ferredoxin reductase did not seem to cause a significant loss of reducing power and the formation of C4 was more preferred compared to the formation of C2 [38]. It was reported that the enzymes involved in NADH oxidization in *C. perfringens* have the following order of affinity: Ldh ⩾ Hdb ⩾ Bcd, EtfA and EtfB complex > AdhE ≫ NADH-ferredoxin reductase. Interestingly, the overexpression of the NADH-dependent *adhE2* gene upregulated other NADH dependent enzymes such as hydrogenase in *C. tyrobutyricum*, suggesting that the further improvement of butanol production could be achieved by redox rebalance [15].

3.2 Process regulators of redox balance

In addition to genetic engineering, process engineering has a great potential to regulate redox balance and butanol production. The additives in fermentation media could increase the butanol production by affecting the environmental oxidation-reduction potential (ORP) and intracellular metabolism through the availability of NADH. For example, the supplement of methyl viologen, neutral red, or whey in medium increased the butanol-acetone production [39,40]. The detoxification effect of furfural was observed by adding glycerol in growth medium, which also increased the intracellular stock of NADH/NADPH [41]. The addition of electron carriers reduced the activities of hydrogenase, and the loss of hydrogen formation led to the electron flow towards butanol, which was essential for the regeneration of $NAD(P)^+$ pool [3]. The biological redox pairs such as cystine/cysteine and oxidized/reduced glutathione can be supplemented to affect the ORP status. Mannitol was also used as carbon source to produce butanol by an engineered *C. tyrobutyricum*, which produced a high butanol titer of 16.0 g/L [6], but the cost to use mannitol as carbon source is high.

4 Energy metabolism

Similar to the dosage effect of NADH, high ATP level can effectively block the butyrate synthesis and direct the carbon flow to the butanol pathway [42]. The butanol synthesis pathway in Clostridia depends on CoA, and the condensation of acetyl-CoA to acetoacetyl-CoA was proved to be reversible thermodynamically [5] and enzymatically [43]. Therefore, artificial ATP accumulation resulted in the formation of a force driving reaction towards butanol in *E. coli* [37] and cyanobacteria [44]. Because more ATP is produced through the acidogenesis pathway, the high level of intracellular ATP is essential to the redirect the metabolic flux towards the solvent formation. The inactivation of *ack* in *C. tyrobutyricum* increased the butyrate formation and further led to a higher ATP synthesis level. On the other hand, the formation of butanol reduced the ATP generation through butyrate pathway (Fig. 1), and also reduced the expression of pyruvate kinases and phosphoglycerate kinase [15]. Therefore, the inactivation of both acetate and butyrate pathways could increase the production and selectivity of butanol but the energy distribution could be challengeable.

It was suspected that a high ATP level contributed to the high butanol tolerance by compensating the damage caused by butanol [45]. The toxicity of butanol is attributed to the impairment of nutrient transportation and the activity decrease of membrane-bound ATPase [46]. The glycolytic limitation was relieved by overexpressing the homologous *pfkA* (6-phosphofructokinase) and *pykA* (pyruvate kinase) in *C. acetobutylicum*, which increased the intracellular concentrations of ATP and NADH [47]. Also, it was suggested that the initial glucose concentration influenced the stoichiometric coefficients of products and the production of ATP for biomass formation [48]. The regeneration of ATP can be achieved through substrate-level phosphorylation (SLP), respiration, or electron transport phosphorylation (ETP) [49]. However, in the middle of electron transport phosphorylation, the membrane-associated Rnf (Rhodobacter nitrogen fixation) complex was not available in *C. acetobutylicum* [3], which may be caused by the severe damage of acid and solvent on the cell membrane.

5 Conclusions

The production of butanol by Clostridia will continue to be an interesting research area in biofuel development [50]. The accumulation of fermentation data and the advances in proteomics technologies allow for a better understanding of the driving force behind the metabolism regulation of carbon, redox and energy. The rebalance of carbon, redox and energy could result in fast cell growth, and high butanol productivity. The effective approaches of metabolic engineering could be developed at multi-levels (gene, pathway, and system) by rationally regulating the metabolism of carbon, redox and energy.

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References

1. Dürre P. Fermentative butanol production. Annals of the New York Academy of Sciences, 2008, 1125(1): 353–362
2. Caspeta L, Buijs N A A, Nielsen J. The role of biofuels in the future energy supply. Energy & Environmental Science, 2013, 6(4): 177–182
3. Lütke-Eversloh T, Bahl H. Metabolic engineering of *Clostridium acetobutylicum*: Recent advances to improve butanol production. Current Opinion in Biotechnology, 2011, 22(5): 634–647
4. Tracy B P, Jones S W, Fast A G, Indurthi D C, Papoutsakis E T. Clostridia: The importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Current Opinion in Biotechnology, 2012, 23(3): 364–381
5. Zheng Y, Li L, Xian M, Ma Y, Yang J, Xu X, He D. Problems with the microbial production of butanol. Journal of Industrial Microbiology & Biotechnology, 2009, 36(9): 1127–1138
6. Yu M, Zhang Y, Tang I, Yang S. Metabolic engineering of *Clostridium tyrobutyricum* for *n*-butanol production. Metabolic Engineering, 2011, 13(4): 373–382
7. Harris L M, Desai R P, Welker N E, Papoutsakis E T. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnology and Bioengineering, 2000, 67(1): 1–11
8. Jang Y S, Lee J Y, Lee J, Park J H, Im J A, Eom M H, Lee J, Lee S H, Song H, Cho J H, Seung D Y, Lee S Y. Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. mBio, 2012, 3(5): 1–9
9. Cooksley C M, Zhang Y, Wang H, Redl S, Winzer K, Minton N P. Targeted mutagenesis of the *Clostridium acetobutylicum* acetone-butanol-ethanol fermentation pathway. Metabolic Engineering, 2012, 14(6): 630–641
10. Cai G, Jin B, Saint C, Monis P. Genetic manipulation of butyrate formation pathways in *Clostridium butyricum*. Journal of Biotechnology, 2011, 155(3): 269–274
11. Lu C. Butanol production from lignocellulosic feedstocks by acetone-butanol-ethanol fermentation with integrated product recovery. Dissertation for the Doctoral Degree. Columbus: The Ohio State University, 2011, 2–25
12. Tummala S B, Welker N E, Papoutsakis E T. Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. Journal of Bacteriology, 2003, 185(6): 1923–1934
13. Nair R V, Papoutsakis E T. Expression of plasmid-encoded *aad* in *Clostridium acetobutylicum* M5 restores vigorous butanol produc-

tion. Journal of Bacteriology, 1994, 176(18): 5843–5846
14. Yu L, Zhao J, Xu M, Dong J, Varghese S, Yu M, Tang I, Yang S. Metabolic engineering of *Clostridium tyrobutyricum* for *n*-butanol production: Effects of CoA transferase. Applied Microbiology and Biotechnology, 2015, published online
15. Ma C, Kojima K, Xu N, Mobley J, Zhou L, Yang S, Liu X M. Comparative proteomics analysis of high *n*-butanol producing metabolically engineered *Clostridium tyrobutyricum*. Journal of Biotechnology, 2015, 193: 108–119
16. Rajagopalan G, He J, Yang K. A highly efficient NADH-dependent butanol dehydrogenase from high-butanol-producing *Clostridium* sp. BOH3. BioEnergy Research, 2013, 6(1): 240–251
17. Kuit W, Minton N P, López-Contreras A M, Eggink G. Disruption of the acetate kinase (*ack*) gene of *Clostridium acetobutylicum* results in delayed acetate production. Applied Microbiology and Biotechnology, 2012, 94(3): 729–741
18. Zhang Y, Yu M, Yang S. Effects of *ptb* knockout on butyric acid fermentation by *Clostridium tyrobutyricum*. Biotechnology Progress, 2012, 28(1): 52–59
19. Lehmann D, Radomski N, Lütke-Eversloh T. New insights into the butyric acid metabolism of *Clostridium acetobutylicum*. Applied Microbiology and Biotechnology, 2012, 96(5): 1325–1339
20. Heap J T, Cartman S T, Pennington O J, Cooksley C M, Scott J C, Blount B, Burns D A, Minton N P. Clostridia: Molecular Biology in the Post-genomic Era. Norfolk: Caister Academic Press, 2009, 179–198
21. Heap J T, Pennington O J, Cartman S T, Carter G P, Minton N P. The ClosTron: A universal gene knock-out system for the genus *Clostridium*. Journal of Microbiological Methods, 2007, 70(3): 452–464
22. Heap J T, Kuehne S A, Ehsaan M, Cartman S T, Cooksley C M, Scott J C, Minton N P. The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. Journal of Microbiological Methods, 2010, 80(1): 49–55
23. Durre P, Bohringer M, Nakotte S, Schaffer S, Thormann K, Zickner B. Transcriptional regulation of solventogenesis in *Clostridium acetobutylicum*. Journal of Molecular Microbiology and Biotechnology, 2002, 4(3): 295–300
24. Lee S Y, Park J H, Jang S H, Nielsen L K, Kim J, Jung K S. Fermentative butanol production by clostridia. Biotechnology and Bioengineering, 2008, 101(2): 209–228
25. Jang Y, Han M, Lee J, Im J A, Lee Y H, Papoutsakis E T, Bennett G, Lee S Y. Proteomic analyses of the phase transition from acidogenesis to solventogenesis using solventogenic and non-solventogenic *Clostridium acetobutylicum* strains. Applied Microbiology and Biotechnology, 2014, 98(11): 5105–5115
26. Yang S, Giannone R J, Dice L, Yang Z K, Engle N L, Tschaplinski T J, Hettich R L, Brown S D. *Clostridium thermocellum* ATCC27405 transcriptomic, metabolomic and proteomic profiles after ethanol stress. BMC Genomics, 2012, 13(1): 336–353
27. Nakayama S, Kosaka T, Hirakawa H, Matsuura K, Yoshino S, Furukawa K. Metabolic engineering for solvent productivity by downregulation of the hydrogenase gene cluster *hupCBA* in *Clostridium saccharoperbutylacetonicum* strain N1-4. Applied Microbiology and Biotechnology, 2008, 78(3): 483–493
28. Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Applied Microbiology and Biotechnology, 2008, 77(6): 1305–1316
29. Grupe H, Gottschalk G. Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Applied and Environmental Microbiology, 1992, 58(12): 3896–3902
30. Berríos-Rivera S. The effect of increasing NADH availability on the redistribution of metabolic fluxes in *Escherichia coli* chemostat cultures. Metabolic Engineering, 2002, 4(3): 230–237
31. Vemuri G N, Eiteman M A, McEwen J E, Olsson L, Nielsen J. Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America, 2007, 104(7): 2402–2407
32. Jo J H, Lee D S, Park J M. The effects of pH on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. Bioresource Technology, 2008, 99(17): 8485–8491
33. Jiang M, Chen J, He A, Wu H, Kong X, Liu J, Yin C, Chen W, Chen P. Enhanced acetone/butanol/ethanol production by *Clostridium beijerinckii* IB4 using pH control strategy. Process Biochemistry, 2014, 49(8): 1238–1244
34. Tsai T, Lo Y, Chang J. Effect of medium composition and pH control strategies on butanol fermentation with *Clostridium acetobutylicum*. Energy Procedia, 2014, 61: 1691–1694
35. Vardar-Schara G, Maeda T, Wood T K. Metabolically engineered bacteria for producing hydrogen via fermentation. Microbial Biotechnology, 2008, 1(2): 107–125
36. Li F, Hinderberger J, Seedorf H, Zhang J, Buckel W, Thauer R K. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. Journal of Bacteriology, 2008, 190(3): 843–850
37. Shen C, Lan E, Dekishima Y, Baez A, Cho K M, Liao J. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Applied and Environmental Microbiology, 2011, 77(9): 2905–2915
38. Yu R, Wang R, Bi T, Sun W, Zhou Z. Blocking the butyrate-formation pathway impairs hydrogen production in *Clostridium perfringens*. Acta Biochimica et Biophysica Sinica, 2013, 45(5): 408–415
39. Wang Y, San K Y, Bennett G N. Cofactor engineering for advancing chemical biotechnology. Current Opinion in Biotechnology, 2013, 24(6): 994–999
40. Du Y, Jiang W, Yu M, Tang I, Yang S. Metabolic process engineering of *Clostridium tyrobutyricum* Δ*ack-adhE2* for enhanced n-butanol production from glucose: Effects of methyl viologen on NADH availability, flux distribution, and fermentation kinetics. Biotechnology and Bioengineering, 2015, 112(4): 705–715
41. Ujor V, Agu C V, Gopalan V, Ezeji T C. Glycerol supplementation of the growth medium enhances *in situ* detoxification of furfural by *Clostridium beijerinckii* during butanol fermentation. Applied Microbiology and Biotechnology, 2014, 98(14): 6511–6521
42. Hüsemann M H, Papoutsakis E T. Comparison between *in vivo* and

in vitro enzyme activities in continuous and batch fermentations of *Clostridium acetobutylicum*. Applied Microbiology and Biotechnology, 1989, 30(6): 585–595

43. Wiesenborn D P, Rudolph F B, Papoutsakis E T. Thiolase from *Clostridium acetobutylicum* ATCC 824 and its role in the synthesis of acids and solvents. Applied and Environmental Microbiology, 1988, 54(11): 2717–2722
44. Lan E, Liao J. ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America, 2012, 109(16): 6018–6023
45. Ventura J S, Hu H, Jahng D. Enhanced butanol production in *Clostridium acetobutylicum* ATCC 824 by double overexpression of 6-phosphofructokinase and pyruvate kinase genes. Applied Microbiology and Biotechnology, 2013, 97(16): 7505–7516
46. Bowles L K, Ellefson W L. Effects of butanol on *Clostridium acetobutylicum*. Applied and Environmental Microbiology, 1985, 50(5): 1165–1170
47. Wang J, Yang X, Chen C, Yang S. Engineering clostridia for butanol production from biorenewable resources: from cells to process integration. Current Opinion in Chemical Engineering, 2014, 6: 43–54
48. Jo J H, Lee D S, Kim J, Park J M. Effect of initial glucose concentrations on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. Journal of Microbiology and Biotechnology, 2009, 19(3): 291–298
49. Herrmann G, Jayamani E, Mai G, Buckel W. Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. Journal of Bacteriology, 2008, 190(3): 784–791
50. Lan E, Liao J. Microbial synthesis of *n*-butanol, isobutanol, and other higher alcohols from diverse resources. Bioresource Technology, 2013, 135: 339–349