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REVIEW

Problems with the microbial production of butanol

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Abstract With the incessant fluctuations in oil prices and increasing stress from environmental pollution, renewed attention is being paid to the microbial production of biofuels from renewable sources. As a gasoline substitute, butanol has advantages over traditional fuel ethanol in terms of energy density and hygroscopicity. A variety of cheap substrates have been successfully applied in the production of biobutanol, highlighting the commercial potential of biobutanol development. In this review, in order to better understand the process of acetone–butanol–ethanol production, traditional clostridia fermentation is discussed. Sporulation is probably induced by solvent formation, and the molecular mechanism leading to the initiation of sporulation and solventogenesis is also investigated. Different strategies are employed in the metabolic engineering of clostridia that aim to enhancing solvent production, improve selectivity for butanol production, and increase the tolerance of clostridia to solvents. However, it will be hard to make breakthroughs in the metabolic engineering of clostridia for butanol production without gaining a deeper understanding of the genetic background of clostridia and developing more efficient genetic tools for clostridia. Therefore, increasing attention has been paid to the metabolic engineering of *E. coli* for butanol production. The importation and expression of a non-clostridial butanol-producing pathway in *E. coli* is probably the most promising strategy for butanol biosynthesis. Due to the lower butanol titers in the fermentation broth, simultaneous fermentation and product removal techniques have been developed to reduce the cost of butanol recovery. Gas stripping is the best technique for butanol recovery found so far.

Keywords Butanol · Fermentation · Metabolic engineering · Clostridia · *E. coli* · Recovery techniques

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Introduction

Diminishing oil resources and incessant fluctuations in oil prices have stimulated efforts towards biosynthesizing fuels from renewable resources [17, 55]. Ethanol, a traditional biofuel, is not an ideal fuel due to its high hygroscopicity and low energy density, which increase the difficulty involved in and expense of its storage and distribution. Compared to ethanol, butanol, which is less hygroscopic and less volatile, has an energy density that is closer to gasoline [10]. In addition, butanol is also used as a chemical feedstock in the plastic and flavor industries [52]. Therefore, increasing attention has been paid to butanol production in recent years.

Butanol can be produced by clostridia fermentation, which used to rank second only to yeast fermentation in terms of scale of production. Clostridia, a group of Gram-positive, spore-forming, obligate anaerobes, naturally possess pathways that allow the conversion of sugar into solvents, known as acetone–butanol–ethnol (ABE) fermentation. A

number of different clostridia have been isolated and their abilities in relation to butanol production verified, but *Clostridium acetobutylicum* is the species that is most often used for the industrial production of butanol. Though its solvent-producing pathways are known, how the metabolic fluxes are regulated is still unclear. In order to obtain deeper insight into the flux control of the butanol pathway, it is necessary to perform a general analysis of the metabolic pathways of *C. acetobutylicum*.

In clostridia fermentation, the sporulation occurs concomitantly with the solventogenesis. Sporulation makes the bacterial cells enter a dormant state where they lose the ability to produce solvents. It is likely that there is a relationship between sporulation and solventogenesis, given that many early molecular events connected with sporulation appear in the initiation of solventogenesis [12, 58, 59]. If this relationship is revealed, it may be possible to produce more solvents, including butanol, by preventing the clostridia from forming spores.

Metabolic engineering, through which the metabolic flux can be directed towards the required products, plays an important role in the development of biofuels, including butanol. Quite a lot of progress in enhancing solvent production, improving the selectivity for butanol, and in increasing the tolerance of bacteria for the solvent has been achieved through the metabolic engineering of clostridia. Metabolic engineering of clostridia has been practiced for many years as the traditional approach used by many research groups. On the other hand, the metabolic engineering of *E. coli* has only recently gained attention for butanol production [2, 61]. Though *E. coli* does not naturally produce butanol, it can be endowed with the ability to produce butanol through metabolic engineering. *E. coli* is an ideal candidate for biofuel production because it grows quickly and genetic tools for its modification are well developed.

So far, the butanol titer achieved has been relatively low. The recovery of low titers of butanol makes the commercial production of butanol more expensive. Therefore, a variety of butanol recovery techniques have been developed to reduce the cost of butanol bioproduction [13, 14, 21, 43, 48].

Here, we review advances and problems in the microbial production of butanol, including clostridia fermentation, sporulation and solventogenesis, the metabolic engineering of clostridia, the metabolic engineering of *E. coli*, and butanol recovery techniques.

Clostridia fermentation

Butanol-producing clostridia such as *C. acetobutylicum*, *C. beijerinckii* and *C. pasteurianum* exhibit very similar metabolic pathways [8, 71, 74]. During fermentation, *C. acetobutylicum* produces three major classes of products: (i) solvents (acetone, ethanol and butanol); (ii) organic acids (acetic acid, lactic acid and butyric acid); (iii) gases (carbon dioxide, and hydrogen). The biosyntheses of acetone, butanol and ethanol share the same metabolic pathway from glucose to acetyl-CoA but branches into different pathways thereafter (Fig. 1). Five enzymes, acetoacetyl-CoA thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase and aldehyde/alcohol dehydrogenase, which are encoded by *thl*, *hbd*, *crt*, *bcd* and *adhE/adhE2*, respectively, are needed to complete the conversion of acetyl-CoA to butanol [1, 3]. Except for the *thl* gene, the butyrate central pathway genes are all located in a cluster [5, 37].

A typical ABE fermentation using *C. acetobutylicum* yields acetone, butanol and ethanol in the ratio of 3:6:1. It seems that acetyl-CoA is mostly used to form butyryl-CoA, based on the fact that the conversion of acetyl-CoA to butyryl-CoA exhibits enhanced thermodynamic stability ($\triangle_r G_m^\theta = -14.2$ kcal/mol) [39, 40]. In the conversion processes above, the condensation reaction of acetyl-CoA to acetoacetyl-CoA may be the rate-limiting step, since this reaction is quite unfavorable thermodynamically ($\triangle_r G_m^\theta = 5.3$ kcal/mol) [39, 40]. A high concentration of acetyl-CoA is needed to make this reaction go smoothly, and the quantity of acetyl-CoA plays an important role in determining the ratio of C3 + C4 products to C2 products [72].

It is likely that ABE fermentation first undergoes an acidogenic phase in the exponential growth phase, and then switches to a solventogenic phase at the end of the exponential growth phase. In the acidogenic phase, more butyrate is produced than acetate, because the formation of butyrate better resolves the issue of redox equilibrium—the NADHs produced during glycolysis are only consumed in butyrate, not in acetate formation pathways. The fact that more butanol than ethanol is produced in the fermentation broth may support this point, since most of the butyrate and acetate convert to butanol and ethanol in the solventogenetic phase, respectively. Through the metabolic engineering of the nonsporulating, nonsolventogenic *C. acetobutylicum*, Sillers et al. [64] verified the importance of electron balance in butanol production. In addition, the reason why acidogenic phase matches well with the exponential growth phase of bacteria fermentation is that acid formation is accompanied by the synthesis of ATP, which is required for cell growth. The butyrate and acetate pathways play important roles in the energy metabolism of clostridia, given that clostridia are obligate anaerobes. When the concentrations of undissociated acids exceed some threshold value, solventogenesis is triggered [38]. Meanwhile, cell growth rate decreases, and the cells enter a stationary phase. Once the concentrations of undissociated acids exceed the threshold value (maybe 57–60 mmol/l), an "acid crash" occurs. Based on this knowledge, Mutschlechner et al. [44] adopted a continuous two-stage ABE fermentation using *C. beijerinckii* NRRL B592. In the first stage, the organism was cultured acidogenically as rapidly as

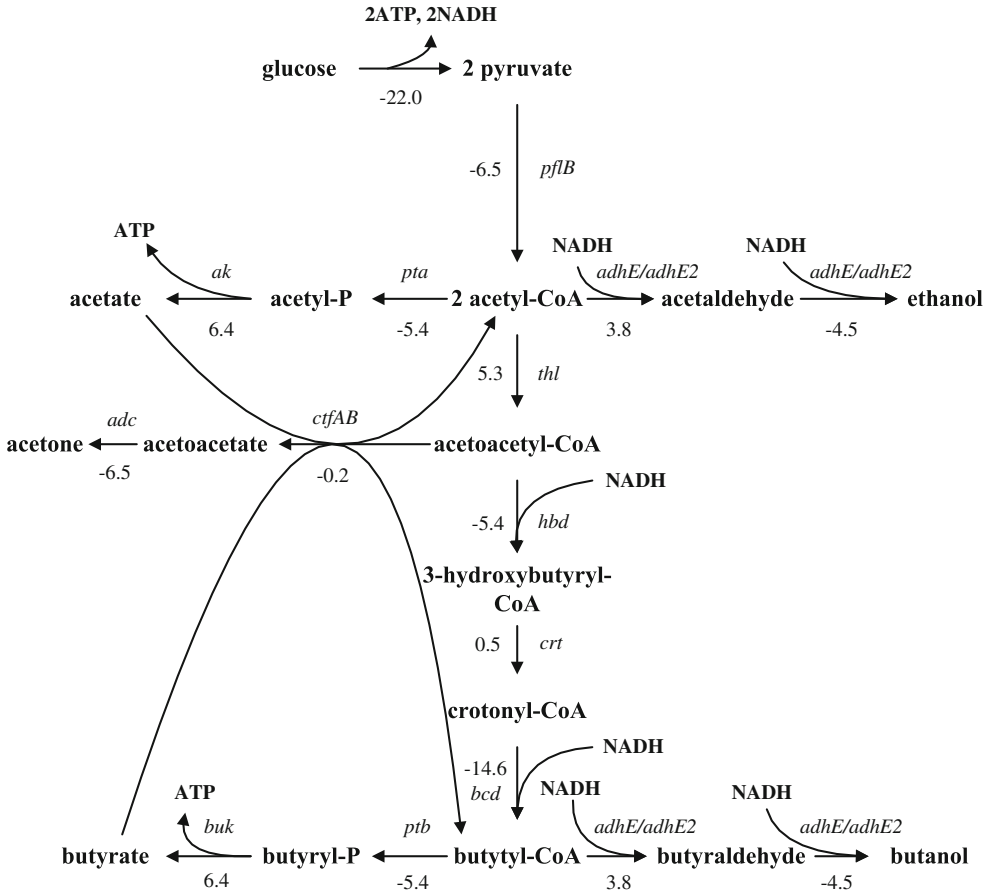

Fig. 1 Metabolic pathways of *C. acetobutylicum*. The numbers shown in the figure represent the standard Gibbs energy changes ($\triangle_r G_m^{\theta}$) of the corresponding reactions. The genes are shown in italics, and their corresponding enzymes are as follows: *pflB*, pyruvate ferredoxin oxidoreductase; *thl*, thiolase; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase; *pta*, phosphotransacetylase; *ak*, acetate kinase; *ptb*, phosphotransbutyrylase; *buk*, butyrate kinase; *ctfAB*, acetoacetyl-CoA:acetate/butyrate:CoA transferase; *adc*, acetoacetate decarboxylase; *adhE/adhE2*, aldehyde/alcohol dehydrogenase

possible, and then transferred to the second stage at the "acid break point" for solventogenesis. Using this approach, an average solvent concentration of 15 g/l was obtained [44]. Buffering/pH control and low-temperature fermentation can also be performed to effectively avoid the "acid crash" [6, 38].

When grown in MP2 medium with 60 mM sodium acetate and 8% glucose, *C. beijerinckii* BA101 produced 20.9 g/l butanol (32.6 g/l total solvents), which represents the highest butanol concentration achieved in batch fermentation [7]. Other work, mainly focusing on adding reductive substrates to the medium, was also done to control the metabolic transition from acids to solvents. The addition of viologen dyes to the medium directed the carbon flow from butyrate to butanol production, concomitant with a decrease in hydrogen evolution [56, 65]. Additionally, more butanol was produced by using a binary substrate mixture of glucose and glycerol than by using glucose alone [71].

Strain degeneration is a general challenge experienced in the fermentation industry. Clostridia degeneration includes the loss of capacity to produce solvents and to sporulate. Kashket and Cao [32] isolated a degeneration-resistant mutant of *C. acetobutylicum* NCIMB 8052; the longevity of the mutant was three times higher than that of the wild type. The insertion of transposon Tn*1545* into the regulatory locus of the clostridial genome contributed to the difference. There was no evidence that the inserted element Tn*1545* was connected with strain degeneration. The disruption of a 486 bp regulatory RNA that could form a stable hairpin structure was speculated to be associated with the reduced tendency to degenerate [36]. Bacteriophage infection is another major problem in ABE fermentation. Among the approaches used to eliminate the negative effect of bacteriophage on clostridia fermentation, the most successful and widespread involves the isolation of phage-resistant mutants or variants [31].

Sporulation and solvetogenesis

The induction mechanisms for solventogenesis and sporulation in *C. acetobutylicum* have several features in

common, so attempts have been made to elucidate the relationship between sporulation and solventogenesis. Solventogenesis may stimulate the bacteria to form spores, since solvent formation creates adverse circumstances for cell growth. Sporulation makes the bacterial cell enter a dormant state, which helps the cell to live through the adverse circumstances.

Various sigma factor genes from *C. acetobutylicum* were cloned and characterized in order to elucidate the molecular mechanism leading to the initiation of sporulation and solventogenesis [12, 58, 59]. Sigma factors are required for RNA polymerases to initiate transcription by recognizing specific promoter sequences. Therefore, they play a central role in controlling mRNA transcription. σ^F, σ^E, σ^G, and σ^K are activated in sequence after septation (with the help of σ^H and Spo0A∼P), and each of these sigma factors participates in the transcription of a specific set of genes and operons [12]. SpoOA∼P can control sporulation by interacting with σ^H. Therefore, SpoOA∼P functions in both sporulation and solventogenesis. It seems that the effect of *spoOA* on solvent formation is part of a balancing act involving the gene expression of sporulation and that of solventogenesis, since spore and forespore-forming cells don't produce solvents. In addition, *abrB310*, which is homologous to the transcriptional factor *abrB*, responsible for sporulation initiation in *Bacillus subtilis*, was identified in *C. acetobutylicum* ATCC 824. It exhibited a transiently elevated level of expression at the onset of solventogenesis, suggesting that it played a positive role in the metabolic transition from acids to solvents [60]. However, the combined effects of these regulatory genes are still not clear, so more investigations are needed to further elucidate the relationship between sporulation and solventogenesis.

Metabolic engineering of clostridia

Genetic tools for metabolic engineering of clostridia

Efficient genetic tools are crucial to the metabolic engineering of clostridia. Because a DNA restriction in *C. acetobutylicum* prevents the efficient expression of recombinant plasmids prepared in *E. coli*, a *B. subtilis*/*C. acetobutylicum* shuttle vector, pFNK1, was constructed for the overexpression of solvent-producing genes in *C. acetobutylicum* [42]. The pFNK1 lacks the sequence 5′-GCNGC-3′, which can be recognized by a restriction endonuclease of *C. acetobutylicum* ATCC 824, so it avoids being cleaved in *C. acetobutylicum* ATCC 824. Metabolic engineering of clostridia is also impeded by the lack of methods for effectively knocking out specific genes. To date, only five genes (*buk*, *pta*, *adhE*, *solR*, *spoOA*) have been knocked out in *C. acetobutylicum*, and four of them were made by single crossover integration of replication-deficient plasmids [24, 25, 27, 45]. Therefore, a universal gene knock-out system for the genus *Clostridium* was developed based on the mobile II intron from the *ltrB* gene of *Lactococcus lactis* (Ll.ltrB) [28].

Promoters play a key role in determining the level of transcription. A reporter gene system is required to study the promoters of specific genes. The *β*-galactosidase gene (*lacZ*) from *Thermoanaerobacterium thermosulfurigenes*, the luciferase gene (*lucB*) from *Photinus pyralis* and the *β*-glucuronidase gene (*gusA*) from *E. coli* have been reported as reporter systems in *C. acetobutylicum*, while a *β*-1,4-endoglucanase gene (*eglA*) from *C. acetobutylicum* P262 was selected as a reporter system in *C. beijerinckii* [20, 22, 53]. These genetic tools facilitated the development of the metabolic engineering of clostridia, which can then be used to enhance solvent production, improve selectivity for butanol production and increase the tolerance of the bacteria to the solvent [34].

Strategies to enhance solvent production

Many endeavors that involve either altering the metabolic regulatory system of *C. acetobutylicum* or overexpressing solvent-producing genes in *C. acetobutylicum* have been made to enhance solvent production. SolR, encoded by *solR*, was once supposed to be a transcriptional repressor with a helix-turn-helix (HTH) motif to bind DNA. The Papoutsakis group [26, 45] found that inactivation of *solR* resulted in the higher levels of butanol and acetone, while overexpression of *solR* resulted in lower levels of butanol and acetone. When overexpression of *aad* was combined with inactivation of *solR*, 17.6 g/l butanol, 8.2 g/l acetone and 2.2 g/l ethanol were produced, which were 51, 66 and 194% higher than the wild-type strain, respectively [26]. However, Thormann and Dürre speculated that SolR (Orf5) might function in glycosylation or deglycosylation, not as a transcriptional repressor, based on the facts that SolR (Orf5) possessed a signal peptide sequence and did not bind to the promoter region upstream of either the *adhE* gene or *adc* gene [66]. The reason why the overexpression of *solR* led to a solvent-negative phenotype in Papoutsakis's study was that, besides the *solR* (*orf5*) gene, the construct Papoutsakis used had a SpoOA-binding motif, and the combination of this with SpoOA eliminated the functionality of SpoOA for transcriptional activation [67].

It is well known that SpoOA is a transcriptional regulator that positively controls sporulation and solvent production [11, 27, 67]. The *spoOA* inactivation strain accumulated less solvents (0.1 and 1.0 g/l, respectively, for acetone and butanol) than the wild-type strain (5.3 and 12.7 g/l, respectively, for acetone and butanol), while the *spoOA* overexpression strain produced a higher concentration of butanol (10.2 g/l) than the plasmid control strain

(9.2 g/l) [27]. Given that DNA of *C. acetobutylicum* becomes less negatively supercoiled at the onset of solventogenesis, SpoOA probably contributes to the decrease in negative supercoiling [73]. Furthermore, SpoOA functions by binding to *ptb* and *adc* promoter OA boxes, resulting in downregulation of *ptb* gene expression and upregulation of *adc* gene expression, which benefits the enhancement of butanol production [57].

C. acetobutylicum ATCC 824 with overexpression of the acetone-formation genes (*adc*, *ctfA*, *ctfB*) produced 13.2 g/l butanol and 8.6 g/l acetone at pH 5.5, final concentrations that were 37% and 90% higher, respectively, than the plasmid-free control strain [41].

To date, most work to increase solvent production has focused on increasing the quantity of enzyme products involved in butanol biosynthesis. However, this approach did not improve butanol production significantly. *C. acetobutylicum* is an obligate anaerobe, so its energy production is rather inefficient. However, protein synthesis is rather energy intensive, so overexpression of related enzymes probably disrupts the biosynthesis of other metabolites that indirectly favor the production of butanol [51]. Therefore, improving the activity of enzyme products by protein engineering may be an alternative method of enhancing solvent production. Rational design and directed evolution (laboratory evolution) are the approaches generally used in protein engineering. Glieder et al. [23] obtained a highly active alkane hydroxylase by directed evolution. The evolved enzyme exhibited a turnover rate that was 20 times higher than the wild type for the selective oxidation of hydrocarbons of small to medium chain length. Therefore, it is feasible to apply this technique to improve the activities of enzymes involved in butanol production.

Strategies to improve the selectivity for butanol production

Selective production of butanol will make product recovery much easier. Tummala et al. [69] adopted an antisense RNA (asRNA) strategy to improve the selectivity for butanol production. However, the acetone and butanol titers (0.3 and 2.6 g/l, respectively) decreased upon downregulating the second CoA transferase gene (*ctfB*) in *C. acetobutylicum*. This demonstrated that asRNA against *ctfB* degraded the whole *sol* operon (*aad-ctfA-ctfB*) transcript. Therefore, a strategy of asRNA against *ctfB* combined with *aad* overexpression was employed to increase the butanol/acetone ratio. Indeed, the butanol/acetone ratio of this engineered strain (4.89 ± 0.29) was more than twofold greater than that of the control strain (1.83 ± 0.05). Though the selectivity of the process for butanol was increased by the metabolic engineering of clostridia, the butanol titer of this engineered strain (ca. 9.8 g/l) was lower than that of the wild type (ca. 10.2 g/l). The reason for this is probably that the conversion of butyrate to butanol requires the participation of CoA transferase. CoA transferase catalyzes the reversible reaction between butyrate/acetoacetyl-CoA and butanoyl-CoA/acetoacetate. Therefore, downregulation of *ctfB* not only blocks the formation of acetoacetate but it also disturbs the synthesis of butyryl-CoA—precursors for acetone and butanol, respectively. Inhibition of *adc* gene expression by antisense RNA was thus used to reduce the formation of acetone [70]. However, effective downregulation of *adc* gene expression didn't lower the level of acetone. Thus, they concluded that coenzyme A-transferase (CoAT), instead of acetoacetate decarboxylase (AADC), was the rate-limiting enzyme in the acetone formation pathway of *C. acetobutylicum*.

Desai et al. [9] also examined the effectiveness of an asRNA strategy for the metabolic engineering of *C. acetobutylicum*. However, they chose *ptb* and *buk* as their target genes instead of the polycistronic *aad-ctfA-ctfB* message. The *ptb* and *buk* genes, which code for phosphotransbutyrylase (PTB) and butyrate kinase (BK), respectively, are connected to butyrate formation. Desai et al. intended to lower the production of butyrate, but, contrary to their expectations, the strain transformed with buk-asRNA produced 34% more butyrate (111 mM) but only 35% more butanol (11.4 g/l) than the control strain (81 mM and 8.4 g/l, respectively, for butyrate and butanol), and the strain transformed with ptb-asRNA produced much less butanol (2.1 g/l) than the control strain (8.4 g/l). These results may be explained by the fact that the butyrate and acetate pathways are essential for the energy metabolism of clostridia. The butyrate or acetate production in clostridia is used to supply the bacterial cells with ATP. The bacteria cannnot obtain sufficient ATP to satisfy the energy needs of cell growth and biosynthesis if the butyrate pathway is blocked. It is probable that butanol is not a desired product for clostridia, and that its production is used to release the stress resulting from low pH conditions.

Mutant M5 of *C. acetobutylicum* ATCC 824 is deficient in butyraldehyde dehydrogenase (BYDH), AADC, and CoAT activity, so it produces neither butanol nor acetone. By overexpressing the *aad* gene, M5 restored butanol production (11.1 g/l at pH 5.75) and produced no acetone [46, 64]. In addition, overexpression of the *thl* gene reduced acetate and ethanol levels, though butanol titers (8.0 and 5.7 g/l, respectively, at pH 5.5 and pH 6.0) were also decreased [64].

Solvent production is closely correlated with the quantity of NADH, and plenty of NADH is critical to producing more butanol and less acetone during clostridia fermentation. In the metabolic pathway of clostridia, H_2-uptake hydrogenase catalyzes the oxidation of H_2, while H_2-evolving hydrogenase catalyzes the evolution of H_2, with concomitant consumption of NADH. Nakayama et al. [47]

adopted an asRNA strategy to downregulate the expression of H_2-uptake hydrogenase genes (*hup CBA*) in *C. saccharoperbutylacetonicum*. The acetone/butanol ratio increased to 0.58 (5.0 acetone, 8.6 g/l butanol) in the antisense strain, compared to 0.27 (3.1 g/l acetone, 11.4 g/l butanol) in the wild-type strain. Therefore, it is possible to increase the butanol production and decrease the acetone production by downregulating the expression of H_2-evolving hydrogenase gene.

In conclusion, the results above demonstrate that downregulation of a single gene is unlikely to control the metabolic flux in the assumed manner, due to the elaborate nature of the metabolic regulatory system of clostridia. Therefore, an effective asRNA strategy to raise the selectivity of the process for butanol should be established based on a better understanding of the genetic background of clostridia.

Strategies to increase the tolerance of clostridia to solvent

It is impossible to accumulate highly concentrated butanol if the butanol-producing bacteria are sensitive to solvents. Therefore, it is important to reduce the sensitivity of the butanol-producing strains to solvents. In *C. acetobutylicum*, overexpression of *groESL*, a class I heat-shock protein gene, resulted in increased tolerance of *C. acetobutylicum* to butanol [68]. The proposed mechanism was that GroEL and GroES, protein products of the *groESL* gene, served to prevent aggregation and assist in protein folding under heat-shock or stress conditions. Narberhaus et al. [49] also identified a cluster of heat-shock genes in the *dnaK* gene region of *C. acetobutylicum*, including *grpE*, *dnaK*, *dnaJ* and a new heat-shock gene coding for an unknown heat-shock protein. DnaK, the protein product of the *dnaK* gene, may aid the tolerance of *C. acetobutylicum* to solvent, given that it is induced during the onset of solventogenesis. The other three heat-shock proteins may have the same function as DnaK, but a further investigation is needed to verify this assumption.

In addition, an induced mutation strategy was also employed to screen for butanol-tolerant mutants [29]. However, their tolerance mechanism is yet to be elucidated, although a butanol-tolerant mutant was found to have the ability to uptake glucose more efficiently through an unconventional nonphosphotransferase system during the solventogenic stage [33]. A change in the method of glucose uptake probably suggests a change in the membrane composition, which is probably connected with the tolerance of clostridia for butanol.

Furthermore, the establishment of a butanol efflux system or the degradation of toxic butanol to a less toxic product also probably contributed to the tolerance of clostridia to butanol [68].

Finding a gene or genes responsible for the tolerance of bacteria to butanol will greatly facilitate the construction of butanol-tolerant strains. Jocob and Eleftherios [4] identified the elements of a genomic library enriched by preferential growth under conditions of butanol stress. Future work will focus on sequencing related genes and characterizing their roles in the tolerance of bacteria for butanol.

Metabolic engineering of *E. coli*

Though the metabolic engineering of clostridia has been developed for many years, increasing attention is now being paid to the metabolic engineering of *E. coli* for butanol production. Atsumi et al. [1] engineered a synthetic pathway in *E. coli* for butanol-specific production. A set of genes (*thl*, *hbd*, *crt*, *bcd*, *etfAB* and *adhE2*) involved in the biosynthesis of butanol in clostridia were cloned and expressed in *E. coli*. The whole 1-butanol production pathway in engineered *E. coli* is described in Fig. 2. 139 mg/l butanol were produced under anaerobic conditions via this synthetic pathway. Deleting some competitive host genes further elevated the butanol titer (373 mg/l). In addition, cultures produced fivefold more butanol (552 mg/l) in TB medium supplemented with glycerol than cultures grown in M9 medium (113 mg/l). However, Boyton et al. [5] detected no BCD activity when the *bcd* gene was cloned into *E. coli.* This result may be attributed to improper folding of the expressed protein and its lack of function in *E. coli*. Inui et al. [30] also cloned and expressed butanol-producing genes of *C. acetobutylicum* ATCC 824 in *E. coli*. Unlike Atsumi et al., they used *adhe1*or *adhe* as a substitute for *adhe2* for the overexpression of butanol dehydrogenase [30]. The *E. coli* strain with *adhe* produced 1.2 g/l butanol, four times as much as the strain with *adhe1*.

The low butanol titer in Atsumi's study may be attributed to the low efficiency of multigene expression. After all, co-overexpression of six genes in the same host is still a challenge. Therefore, it is difficult to increase the butanol titer by overexpressing the butanol-producing pathway of clostridia in *E. coli* unless an effective multigene expression technique is developed. Future work could focus on the coordination of multigene expression and optimizing the culture conditions to achieve higher butanol titers.

Recently, instead of using the naturally evolved pathway for butanol production in clostridia, the research group of James C. Liao [2, 61] utilized the highly active amino acid biosynthetic pathway of *E. coli* for alcohol synthesis (Fig. 3). 2-Keto acids, intermediates in amino acid biosynthesis pathways, can be converted to alcohols by overexpression of 2-keto acid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs) in *E. coli*. However, 2-ketovalerate, a 2-keto acid precursor for 1-butanol, is not a common

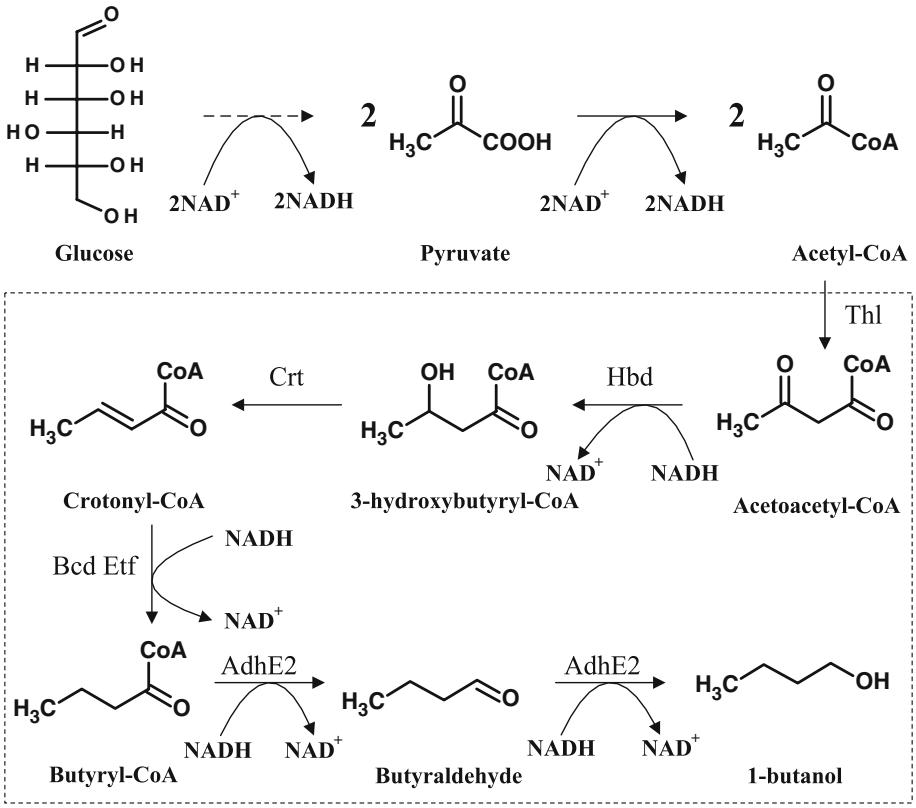

Fig. 2 Schematic representation of 1-butanol production in engineered *E. coli* employing the butanol production pathway from *C. acetobutylicum* (shown in the *dashed box*). The engineered pathway from acetyl-CoA to 1-butanol involves five enzymes, which are abbreviated to: Thl, acetoacetyl-CoA thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; AdhE2, aldehyde/alcohol dehydrogenase

metabolite in *E. coli*. Therefore, an operon encoding the IlvA-LeuABCD pathway needs to be cloned into *E. coli*. Threonine dehydratase, the product of the *ilvA* gene, catalyzes the reaction of *L*-threonine to 2-ketobutyrate, and the protein product of the *leuABCD* gene favors the conversion of 2-ketobutyrate to 2-ketovalerate. The strain with overexpression of the *ilvA-leuABCD* pathway produced ~5-fold more butanol (ca. 30 mg/l) than the strain without overexpression of this pathway [61]. Overexpression of *ilvA* and *leuABCD* led to a higher metabolic flux to 2-ketobutyrate and utilized norvaline synthetic chemistry for the major 2-ketovalerate biosynthesis route [61]. Threonine production was found to be the bottleneck in alcohol accumulation, so a feedback-resistant ThrA ($ThrA^{fbr}$) operon $thrA^{fbr}BC$ was cloned and overexpressed, resulting in three- to fourfold higher titers of 1-propanol (ca. 175 mg/l) and 1-butanol (ca. 100 mg/l). By further knocking out the host genes *metA* and *tdh*—coding for homoserine O-succinyltransferase and threonine dehydrogenase, respectively—the combined production of 1-propanol and 1-butanol increased to 1.2 g/l, with the main contribution coming from 1-propanol. However, interruption of the valine, leucine and isoleucine biosynthesis pathway led to a twofold higher level of butanol (ca. 0.8 g/l), but had little effect on propanol. The improvement in selectivity was probably due to the increased availability of 2-ketobutyrate and acetyl-CoA, the release of LeuABCD from their native substrates, and the relief of IlvA and LeuA by blocking the biosynthesis of leucine and isoleucine [61].

Construction of a nonclostridial butanol-producing pathway used for butanol biosynthesis is probably the most promising strategy. For example, *E. coli* is famous for its rapid growth; it exhibits great advantages in the selective production of butanol, and possesses a much higher efficiency of energy production, which effectively supports the overexpression of exogenous genes in *E. coli*. In addition, given that we understand the genetic background of *E. coli* well, it is possible to design better engineered strains for butanol production. However, there are still technical challenges for metabolic engineering in this case, such as increasing the titer of butanol.

Butanol recovery techniques

Though many attempts have been made to improve butanol production, the concentrations of butanol in the fermentation broth are not yet satisfactory. The recovery of low concentrations of butanol by traditional distillation is energy intensive and thus economically infeasible. Therefore, simultaneous fermentation and product removal techniques, including adsorption, liquid–liquid extraction, perstraction, reverse osmosis, pervaporation and gas stripping, have been developed to reduce the cost of butanol recovery [13, 18, 21, 48, 54].

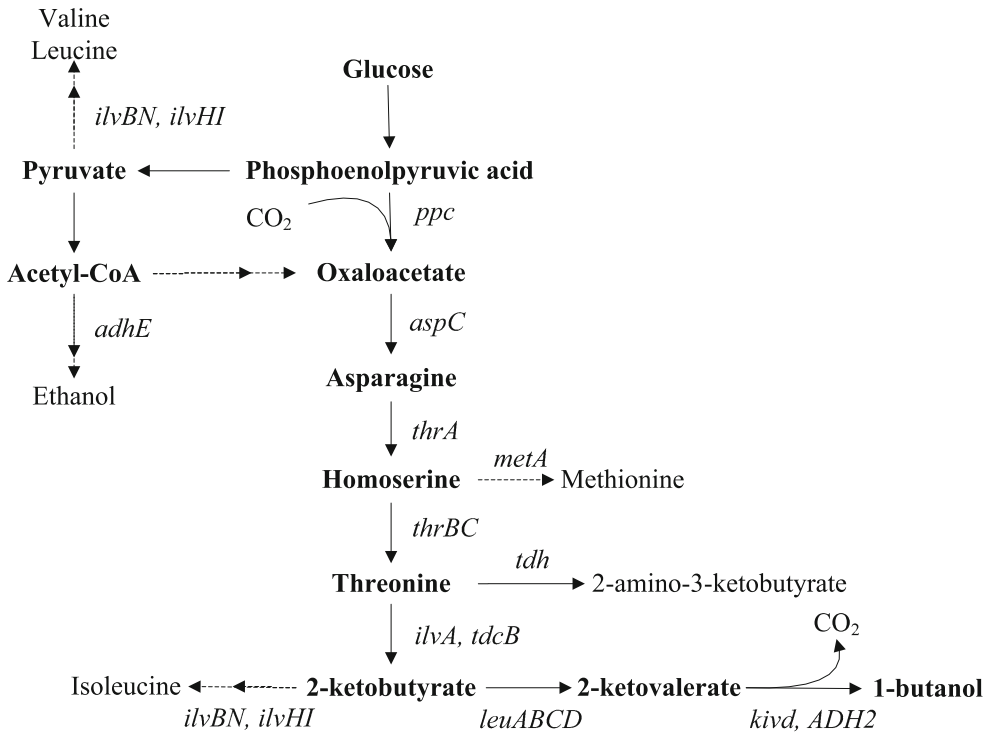

Fig. 3 Schematic representation of 1-butanol production via the norvaline biosynthetic pathways in the engineered *E. coli*. Pathways leading to the biosynthesis of 1-butanol are shown in *bold*. Other products connected with the butanol-producing pathway are indicated in *regular typeface*. All genes are shown in *italics*

Adsorption

Adsorption is a simple technique that can be used to remove butanol from the fermentation broth energy efficiently. In this manner, butanol is first adsorbed by adsorbents from the fermentation broth and then desorbed by heat treatment or displacers to give concentrated butanol solutions as final products. A variety of materials can be used as adsorbents for butanol recovery, but silicalite is the one used most often [18]. Silicalite, a form of silica with a zeolite-like structure and hydrophobic properties, can selectively adsorb small organic moleculars like C_1–C_5 alcohols from dilute aqueous solutions. Adsorbing 1-butanol from a 0.5% solution, drying the silicalite at 40°C, and then heating to 150°C resulted in a condensate containing 98% (w/v) butanol [43].

Liquid–liquid extraction

Liquid–liquid extraction is considered to be an important technique for butanol recovery. Usually, a water-insoluble organic extractant is mixed with the fermentation broth. Butanol is more soluble in the extractant phase than in the fermentation broth phase; therefore, butanol selectively concentrates in the organic phase. As the extractant and fermentation broths are immiscible, the extractant can easily be separated from the fermentation broth after butanol extraction. It should be noted that liquid–liquid extraction is able to extract butanol from the fermentation broth without removing substrates, water or nutrients. Unfortunately, liquid extractants with high butanol distribution coefficients are toxic to the culture, while nontoxic liquid extractants have low butanol distribution coefficients. Therefore, Evans et al. [13] used mixed extractants to balance both pitfalls. The mixed extractant that contained 20% toxic decanol in nontoxic oleyl alcohol enhanced butanol production by 72% under pH-controlled conditions [13]. Shi et al. [62] formulated a general mathematical model to evaluate the performance of an AB continuous flash extractive fermentation system. Improvements in terms of productivity, energy utilization efficiency and product purity may benefit from the mathematical formulation and analysis approach.

Perstraction

Several problems are associated with liquid–liquid extraction, such as cell toxicity, loss of extraction solvent, the formation of an emulsion, and the accumulation of microbial

cells at the extractant and fermentation broth interphase. To solve these problems, a recovery technique called perstraction was developed. In a perstractive separation, the extractant and the fermentation broths are separated by a membrane, which allows butanol to diffuse into the extractant phase. The existence of the membrane greatly reduces, if not eliminates, the toxicity of the extractants, but the rate of butanol extraction is limited, because the membrane presents a physical barrier between the extractant phase and the fermentation broth [18].

Reverse osmosis

Reverse osmosis is another recovery technique that relies on membranes. It is necessary to remove the suspended vegetative organisms using a hollow-fiber ultrafilter before the reverse osmosis is carried out. After the pretreatment, reverse osmosis starts to dewater the fermentation liquor by rejecting solvents but allowing water to pass through the membrane. Consequently, the products are concentrated, and the volume of liquid to be distilled is dramatically reduced. Garcia et al. [21] reported that polyamide membranes exhibited rejection rates as high as 98%, and that optimum rejection of butanol in the fermentation liquor occurred at recoveries of 20–45%.

Pervaporation

Pervaporation is a technique that allows the selective removal of volatile compounds from the model solution/fermentation broth using a membrane. A concentrate and vapor pressure gradient is used to allow one component to preferentially permeate across the membrane [35]. A vacuum applied to the side permeated to is coupled to the immediate condensation of the permeated vapors. Pervaporation functions independent of the vapor/liquid equilibrium, and the permeate must be volatile under the operating conditions. Pervaporation is typically suited to separating a minor component of a liquid mixture, so high selectivity through the membrane is essential. Polydimethylsiloxane membranes and silicon rubber sheets are generally used for the pervaporation process. In addition, a silicalite membrane for ethanol recovery was recently reported that exhibited high selectivity towards ethanol [48]. A further investigation is needed to confirm whether it possesses high selectivity towards butanol or not, since the selectivity of the same membrane towards different solvents varies. Advantages of pervaporation include low energy consumption, entrainer is not required, and no contamination. However, the membrane needs to be regenerated at some point, because a "swelling" effect makes the membrane more permeable but less selective when material passes through the membrane.

Gas stripping

Among various recovery techniques, gas stripping is a promising technique that can be applied to butanol recovery during ABE fermentation. Gas stripping allows for the selective removal of solvents from fermentation broth and uses no membrane, since membrane-based recovery systems can suffer from fouling and clogging. The production of ABE is associated with the generation of gases (H_2 and CO_2). Gases can be transferred into the bioreactor through a sparger, which creates bubbles. When bubbles are formed or broken in the bioreactor, the surrounding liquid vibrates, resulting in the removal of butanol from the fermentation broth. The vaporized butanol is subsequently condensed and separated from the condenser. Once the vaporized butanol is condensed, the carrier gas is recycled back to the bioreactor to capture more butanol [14]. When product recovery is combined with gas stripping, butanol-producing clostridia can utilize concentrated sugar solutions for butanol production. Ezeji et al. [16] investigated the production of butanol in an integrated fed-batch fermentation-gas stripping product-recovery system using *C. beijerinckii* BA101, with H_2 and CO_2 used as the carrier gases. They employed a 500 g/l concentrated glucose solution during fermentation, and achieved 13-fold more solvents (232.8 g l^{-1}) and fourfold higher productivity (1.16 g/l/h) in this integrated system than in the nonintegrated control system. Ezeji [19] also studied the integrated system mentioned above using liquefied corn starch as substrate, and reported that 81.3 g/l ABE was produced, compared to 18.6 g/l for the nonintegrated control system. Factors such as gas recycle rate, bubble size, presence of acetone and ethanol in the broth, which may affect the performance of a recovery system based on gas stripping, were investigated to improve the product recovery performance [14]. It was found that, among the factors tested, the gas recycle rate and the amount of antifoam added influenced the butanol recovery system.

In summary, gas stripping appears to be superior to other product recovery techniques since it does not harm the culture, remove the nutrients and intermediates, require expensive extractants, or rely on membrane technology [15, 54].

Conclusion

The relatively high cost of fermentation substrates is one key factor that prevents the commercial application of the microbial production of butanol. Indeed, the cost of substrate poses a problem to the microbial production of butanol. Low-cost substrates, such as algal biomass, excess sludge, palm oil, soy molasses, etc., have been investigated for butanol bioproduction; however, further research into

how to utilize these substrates more effectively is needed. There is no doubt that lignocellulose is potentially the best substrate for butanol production, and more efficient bioconversion of cellulose and hemicellulose is crucial to the economic success of the industrial production of butanol.

The enzymes responsible for butanol production should be characterized in order to deepen our understanding of the thermodynamics and kinetics of the metabolic pathway [63].

The strategy that genetically modifies the clostridia to improve butanol production has been practiced for many years. Although all of the genes involved in the butanol-producing pathway have been cloned, the mechanism of how they are regulated in vivo is still unclear [1]. The challenges of this strategy are a lack of efficient genetic tools and the uncertain regulatory mechanism of ABE fermentation. Due to the little-known, complicated metabolic system of clostridia, it will be hard to make a breakthrough in the metabolic engineering of clostridia for butanol production before we gain a detailed knowledge of its metabolic regulatory network. Attractively, nonfermentative pathways for the biosynthesis of butanol have great potential to achieve high butanol titers, such as the diversion of the 2-keto acid intermediate of the amino acid biosynthetic pathway for butanol production [2, 61]. In addition, the complete whole-genome sequencing of the butanol-producing bacterium *C. acetobutylicum* ATCC 824 facilitates the optimization of metabolic engineering and the subsequent development of novel and efficient organisms for butanol production [50].

Among various simultaneous fermentation and product removal techniques, gas stripping appears to be the most promising. It is capable of effectively increasing the productivity of butanol and reducing the cost of product recovery. Further optimization of its operating conditions will better favor butanol production.

With the development of genomics, molecular systems biology and metabolic engineering, as well as process techniques, biobutanol will be successfully reapplied to commercial production.

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