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Research Article

Metabolic Engineering of *Clostridium cellulovorans* to Improve Butanol Production by Consolidated Bioprocessing

Zhiqiang Wen, Rodrigo Ledesma-Amaro, Minrui Lu, Mingjie Jin,* and Sheng Yang*

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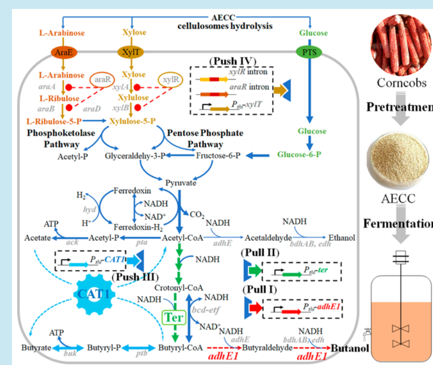
ABSTRACT: *Clostridium cellulovorans* DSM 743B can produce butyrate when grown on lignocellulose, but it can hardly synthesize butanol. In a previous study, *C. cellulovorans* was successfully engineered to switch the metabolism from butyryl-CoA to butanol by overexpressing an alcohol aldehyde dehydrogenase gene *adhE1* from *Clostridium acetobutylicum* ATCC 824; however, its full potential in butanol production is still unexplored. In the study, a metabolic engineering approach based on a push−pull strategy was developed to further enhance cellulosic butanol production. In order to accomplish this, the carbon flux from acetyl-CoA to butyryl-CoA was pulled by overexpressing a trans-enoyl-coenzyme A reductase gene (*ter*), which can irreversibly catalyze crotonyl-CoA to butyryl-CoA. Then an acid reassimilation pathway uncoupled with acetone production was introduced to redirect the carbon flow from butyrate and acetate toward butyryl-CoA. Finally, xylose metabolism engineering was implemented by inactivating *xylR* (*Clocel_0594*) and *araR* (*Clocel_1253*), as well as overexpressing *xylT* (*CA_C1345*), which is expected to supply additional carbon and reducing power for CoA and butanol synthesis pathways. The final engineered strain produced 4.96 g/L of *n*-butanol from alkali extracted corn cobs (AECC), increasing by 235-fold compared to that of the wild type. It serves as a promising butanol producer by consolidated bioprocessing.



KEYWORDS: *consolidated bioprocessing, butanol, Clostridium, push−pull strategy, carbon flux*

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Butanol is an important industrial chemical as well as a renewable promising gasoline substitute.[1] *n*-Butanol is traditionally produced by solventogenic clostridia such as *Clostridium acetobutylicum* or *C. beijerinckii* with corn as feedstock.[2] Recently, butanol production using inexpensive, renewable lignocellulosic biomass has attracted scientific attention due to increasing concern over fossil fuel depletion and the rising price of food resources.[3,4] The main cost of cellulosic butanol fermentation comes from the cellulases used to saccharify lignocellulose, which are required because the butanol-producing clostridia cannot efficiently degrade lignocellulose. Cellulolytic clostridia such as *C. thermocellum*,[5] *C. cellulolyticum*,[6] and *C. cellulovorans*[7] can natively secrete cellulases and grow on lignocellulosic biomass directly having alcohols and organic acids as main products. They offer the possibility to develop consolidated bioprocessing (CBP) that combines cellulase production, lignocellulose hydrolysis, and cofermentation of hexose/pentose to butanol in one step.[8,9]

In the postgenome era, synthetic biology techniques provide many resources and approaches for CBP construction and optimization. Genetic manipulation techniques applicable in cellulolytic clostridia such as TargeTron and the CRISPR/Cas system paved the way for metabolic engineering to produce *n*-butanol and isobutanol.[10−12] *C. cellulolyticum* has been modified to introduce a CoA-dependent metabolic pathway for *n*-butanol production, but the titer was less than 0.1 g/L,[13] partially because this organism lacks a native metabolic pathway from acetyl-CoA to butyryl-CoA. The main limitation to achieve this is that to reconstruct such a long metabolic pathway is burdensome for the host. Alternatively, *C. cellulolyticum* is modified to extend a 2-keto acid metabolic pathway toward isobutanol synthesis, which resulted in 0.66 g/L of isobutanol.[14] Recently, *C. thermocellum*, one of the most effective bacteria for degrading cellulose, was also engineered for isobutanol synthesis,[15] achieving a final titer of 5.4 g/L of isobutanol production from cellulose within 75 h. However, it is difficult to recruit and refine heterologous heat-resistant genetic components that work in thermophilic microorganisms, which explains to some extent why 12 different enzyme combination tests are necessary to reconstruct a hybrid CoA-dependent *n*-butanol synthesis pathway with high titer and thermostability in *C. thermocellum*.[16]

In contrast to *C. cellulolyticum* and *C. thermocellum*, *C. cellulovorans* DSM 743B harbors a complete *n*-butanol pathway according to the prediction of KEGG.[7,17] Interestingly, it can

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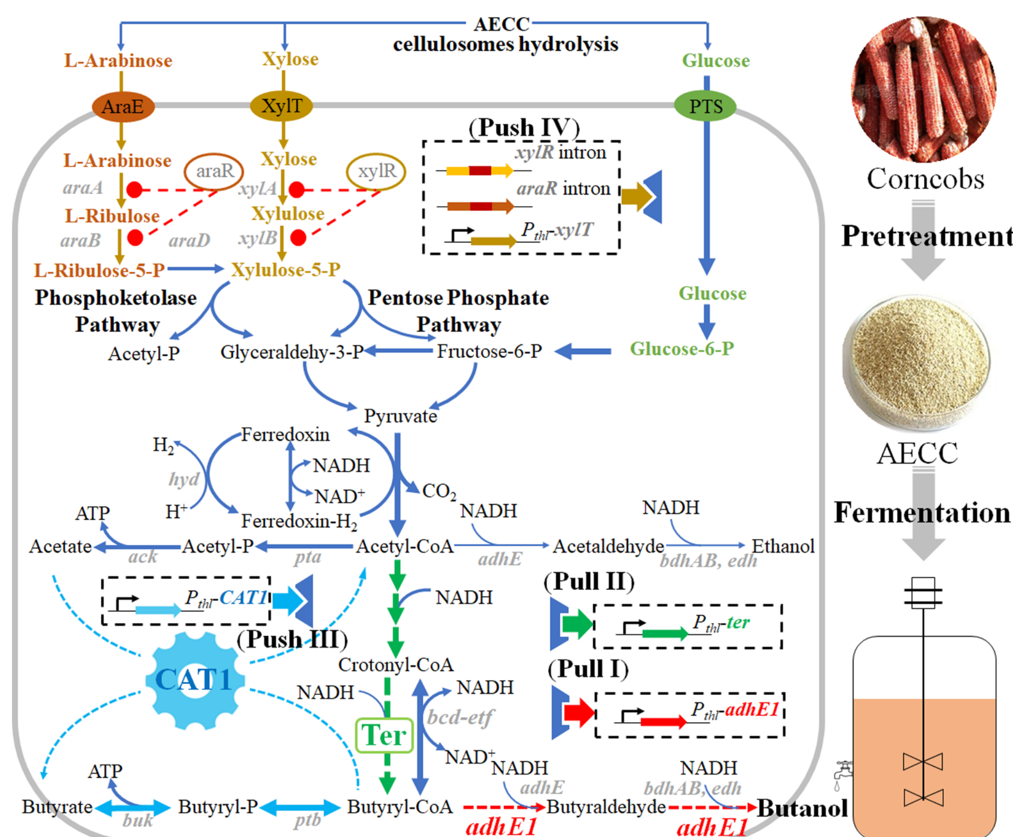

Figure 1. Detailed pull−push modules metabolic engineering approaches of *C. cellulovorans* DSM 743B to enhance butanol production from alkali extracted corn cobs (AECC). Module Pull I pulled carbon flow from butyryl-CoA to butanol synthesis pathway (red) by overexpressing *adhE1*. Module Pull II pulled carbon flow from acetyl-CoA to CoA-dependent (carbon chain elongation) pathway (green) by overexpressing *ter*, which can irreversibly catalyze crotonyl-CoA to butyryl-CoA. Module Push III pushed the carbon flow from butyrate and acetate back to butyryl-CoA and acetyl-CoA by introducing *CAT1* (encoding a butyryl-CoA−acetate CoA transferase) from *C. tyrobutyricum* DSM 2637. Module Push IV improved xylose utilization and thus drove carbon and reducing power to CoA-dependent (carbon chain elongation) pathway (green) and butanol synthesis pathway (red) by inactivating *xylR* and *araR*, as well as overexpressing *xylT*. AraE, arabinose-proton symporter; araA, L-arabinose isomerase; araB, ribulokinase; araD, L-ribulose-5-phosphate 4-epimerase; araR, arabinose utilization negative regulator; XlyT, xylose proton-symporter; xylA, xylose isomerase; xylB, xylulokinase; xylR, xylose utilization negative regulator; PTS, phosphotransferase system; *hydA*, ferredoxin hydrogenase; *ack*, acetate kinase; *pta*, phosphotransacetylase; *adhE* or *adhE1*, acetaldehyde/ethanol dehydrogenase; *bdhA/B*, butanol dehydrogenase; *edh*, alcohol dehydrogenase; *thl*, acetyl-CoA acetyltransferase; *hbd*, beta-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase; *buk*, butyrate kinase; *ptb*, phosphate butyryl-transferase.

hardly synthesize *n*-butanol but can produce over 10 g/L of butyric acid, the precursor of *n*-butanol.[18,19] Yang et al. performed some pioneering and fundamental study of *C. cellulovorans*, including restriction−modification system (R&M system) identification and preliminary metabolic engineering attempts;[20−22] however, further in-depth research was severely delayed due to the lack of efficient genetic tools. More recently, Wen et al. developed a genetic engineering system of the strain and verified that genetic tools such as TargeTron and the CRISPR/Cas system were applicable in *C. cellulovorans*.[23] Based on these efficient genetic techniques, a CoA-dependent acetone−butanol−ethanol (ABE) pathway was introduced in *C. cellulovorans* to redirect the carbon flux to *n*-butanol. In order to further improve the production of *n*-butanol, a strain evolved to tolerate up to 12 g/L of butanol was adapted as host for the above metabolic modification.[17] The generated strains produced 3.47 g/L of butanol with alkali extracted corn cobs (AECC) as sole carbon source, which is 139-fold of that produced by the wild type. This strain also produced 0.51 g/L acetone as byproduct, decreasing butanol yield and increasing the separation cost.[24] The potential of butanol production uncoupled with acetone synthesis has not been systematically examined in *C. cellulovorans*.

Metabolic engineering based on the push−pull strategy is an effective way to unleash the potential of bioproduction.[25−27] Increasing substrate utilization efficiency and reassimilating byproducts are important driving forces, which can push carbon fluxes toward central metabolism and thereby target metabolic products. In addition, enhancing the flux in the pathway of interest by overexpressing key enzymes or by replacing the reversible enzyme with irreversible ones can also effectively pull metabolic flux toward target products. Such push−pull strategy has been applied in enhanced isoprene biosynthesis in *Saccharomyces cerevisiae*,[25] heterologous deoxyviolacein production in *Escherichia coli*,[27] and improved lipid biosynthesis in *Yarrowia lipolytica*,[26] which suggests that the strategy is broad enough to be used for metabolic pathway optimization of *C. cellulovorans*.

According to the theoretical metabolic network of *C. cellulovorans* (Figure 1), acetyl-CoA and butyryl-CoA are two important nodes that link the carbon central metabolism and the butanol synthesis pathway with the CoA-dependent (carbon chain elongation) pathway. Moreover, these nodes

are also closely related to the formation and reassimilation of the byproducts (acetate and butyrate). Based on the available knowledge, several strategies were adapted here to enhance the bottleneck pathway to improve butanol production, as shown in Figure 1. In the previous study, we successfully pulled the metabolism from butyryl-CoA to butanol by overexpression of an alcohol aldehyde dehydrogenase gene *adhE1* from *C. acetobutylicum* ATCC 824.[17] In the present study, we further pulled the carbon flux from acetyl-CoA to butyryl-CoA by overexpressing a codon optimized gene *ter* (encoding a trans-enoyl-coenzyme A reductase, optimal sequence shown in Supporting Information) from *Treponema denticola*,[28,29] which can irreversibly catalyze crotonyl-CoA to butyryl-CoA. In order to prevent the carbon flux from overflowing from acetyl-CoA and butyryl-CoA to acetic acid and butyric acid, respectively, we overexpressed *CAT1* (*CTK_C06520*, encoding a butyryl-CoA−acetate CoA transferase) from *C. tyrobutyricum* DSM 2637,[30,31] which is expected to redirect the carbon flow back to acetyl-CoA and butyryl-CoA. Moreover, the xylose assimilation pathway was engineered to promote xylose utilization by inactivating XylR (a xylose utilization negative regulator, encoded by *Clocel_0594*) and AraR (an arabinose utilization negative regulator, encoded by *Clocel_1253*), as well as overexpressing xylT (a xylose symporter, encoded by *CA_C1345*).[32] The enhanced supply of carbon and reducing power generated in this approach provided important driving forces for the CoA-dependent pathway and the butanol synthesis pathway. The above strategies were partitioned into four individual modules, as shown in Figure 1. Every module was tested independently, and finally they were assembled to maximize butanol production.

METHODS AND MATERIALS

Strains, Plasmids, Primers and Strains Cultivation. All strains, plasmids, and primers used in the study are listed in Table S1 and Table S2. *Escherichia coli* DH5α and ER2275 were aerobically cultivated at 37 °C in liquid or solidified Luria−Bertani (LB) medium, composing of 5 g/L of yeast extract (AngelYeast Co., Ltd., China), 10 g/L of tryptone (AngelYeast Co., Ltd., China), and 10 g/L of NaCl, and 1.5% agar (w/v) if necessary.

C. cellulovorans DSM 743B, *C. tyrobutyricum* DSM 2637, and *C. acetobutyricum* ATCC 824 were cultivated in an anaerobic chamber (AW400SG, Electro-Tech, Co., Ltd., UK) at 37 °C in liquid or solidified Clocel medium with glucose with carbon source as previously described.[17]

All stock culture was maintained in 25% glycerol and frozen at −80 °C.

DNA Manipulation and Vectors Construction. All oligonucleotide primers synthesis and DNA sequencing were conducted by GenScript Biotech Corp (Nanjing, China). All recombinant DNA manipulations including genomic DNA preparation, DNA fragment amplification, digestion, ligation, recombination, transformation, colony PCR, plasmid extraction and identification are performed using the standard procedures previously described.[33,34]

The construction of TargeTron knockout plasmid derived from pWJ1, and all overexpression plasmids derived from pXY1-P_{thl} were accomplished according to annotation for corresponding plasmids and primers in Table S1 and Table S2.

Generation and Screening of Positive Mutants. The transformation procedure of *E. coli* and *C. cellulovorans* DSM 743B was performed as previously described.[17] DNA polymerase easy Taq (TransGen Biotech Co., Ltd., China) or KOD FX Neo (TOYOBO, Co., Ltd., Japan) was used for colony PCR. The products were sequenced as needed by GenScript Biotech Corp (Nanjing, China). Especially, for transformants generated from electro-transformation of pWJ1-*araR* and pWJ1-*xylR*, PCR products of the desired mutants were about 0.9 kb longer than the wild type due to the group II intron insertion. In order to reconfirm the mutants, the PCR products were then sent to GenScript Biotech Corp (Nanjing, China) for sequencing.[17] Curing plasmids used to TargeTron inactivation were accomplished by repeated subculture on solidified medium without erythromycin until we can isolate a single colony sensitive to erythromycin.

Fermentation Profile Investigation of *C. cellulovorans*. *C. cellulovorans* was anaerobically precultured in 200 mL of Clocel medium with AECC as the sole carbon source under static conditions at 37 °C for 24 h before inoculation. The inoculum size of *C. cellulovorans* DSM 743B was 10% (v/v) if not otherwise indicated.[35]

The fermentations used for phenotype comparison were carried out in 500 mL anaerobic shaken flasks (customized from Guxin biotech co., Ltd., Shanghai, China) with a 400 mL working volume with glucose or AECC as sole carbon source. Their data were expressed as bar charts.

As for the fermentations used for metabolic progress investigation, they were accomplished in a 3 L BioFlo 110 bioreactor (New Brunswick Scientific Co., Inc., New Jersey, America) with a 1.6 L working volume. The data were expressed as line chart.

For all fermentation of *C. cellulovorans* DSM 743B, pH is controlled at 7.0 in the first 48 h and then reduced until 6.0 by the automatic addition of 5 N NaOH.[17] The stirring speed and temperature were kept at 150 rpm and 37 °C, respectively. Fifteen mg/L erythromycin was added to the liquid medium to help mutants to harbor pXY1 series plasmids. Samples were collected in an anaerobic chamber (AW400SG, Electro-Tech, Co., Ltd., UK) at regular intervals for analysis of gene transcriptional level, enzyme activity, biomass, substrate, and product concentration.

Enzyme Activity Assay. The assay of butyraldehyde dehydrogenase and butanol dehydrogenase activities in wild strain and 743B(pXY1-P_{thl}-adhE1) were accomplished according to the protocol previously described by Yang et al.[22] except that the crude cell extract was obtained using a lysis method previously described by Shen et al.[28]

The Ter and Bcd activities for crotonyl-CoA were measured using the methods that have been reported by Shen et al. and Li et al.[28,36] For Ter, the reaction mixture contained 100 mM potassium phosphate buffer, pH 6.2, 200 μM NADH, 200 μM crotonyl-CoA, and crude extract.[28] For Bcd, the assay mixture contained 50 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.1 mM NADH, 0.1 mM crotonyl-CoA, 5 μM FAD, 20 μM ferredoxin.[36] The reaction was initiated by the addition of the crude cell extract.

One enzyme activity unit was defined as the oxidation of 1 mol NADH per min. Protein concentration of the crude cell extract is determined using a Thermo Scientific Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, USA).

Real-Time PCR. Real-time PCR is adapted to measure the relative expression level of *ptb* (*Clocel_3675*) and *bk* (*Clocel_3674*) in wild and mutant strain with 16s rRNA gene as control. Total RNA extraction and cDNA preparation

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are performed as previously described.[23] A MyiQ2 two-color real-time PCR detection system (Bio-Rad) and A SYBR Premix Ex Taq kit (Takara, Shiga, Japan) were used for Realtime-PCR. For *ptb* gene (*Cloel_3675*), amplification using the forward primer 5′-ATTTATCGTTGACCCAGTT-3′ and reverse primer 5′-CCATCTAATGCGTTGTTT-3′ resulted in a 246 bp fragment. For *bk* gene (*Cloel_3674*), amplification using the forward primer 5′-TGTTAATACAACTGGTGC-3′ and reverse primer 5′-TGTTAATACAACTGGTGC-3′ resulted in a 155 bp fragment. For the 16S rRNA gene of *C. cellulovorans*, amplification with the forward primer 5′-ACGGCTTTGAGGTGACAGGA-3′ and reverse primer 5′-ACCGAACTAACAATAAGGGTTGC-3′ gave a 105 bp fragment.

Analysis. Biomass in liquid medium was quantified by measuring optical density (OD) at 600 nm using a UV−visible spectrophotometer (TU-1810, Beijing pushen general instrument co., LTD). Biomass in AECC medium was measured according to cell dry weight-protein correlation as previously described.[37] The AECC concentration was calculated by subtracting the estimated cell mass from the pellet dry weight. The concentration of total sugars was estimated using the phenol−sulfuric acid method.

The amounts of sugars (glucose, xylose, cellobiose, arabinose) of fermentation samples were analyzed by a HPLC system (1200 series; Agilent, Wilmington, DE, USA) equipped with a Biorad Aminex HPX-87H column and a refractive index detector. The Aminex HPX-87P column was maintained at 30 °C, and the mobile phase was 0.005 M sulfuric acid at 65 °C with a flow rate of 0.6 mL/min.[38] All liquid samples were filtered and kept refrigerated at 4 °C until analyzed. Concentrations of sugars were calculated based on the calibration sugar standards and shown as an average value of three parallel samples. Volatile solvents (ethanol, butanol, and acetone) and organic acids (acetate and butyrate) were determined by a gas chromatograph (GC-2018-plus, Shimadu co., LTD, Japan) equipped with a HP-INNOWAX (19091N-113) capillary chromatographic column.

Reactor productivity was estimated as butanol production in g/L divided by the fermentation time and was expressed as g/L/h. Butanol yield was calculated as gram of produced butanol per gram of decomposed AECC. The carbon recovery of C2 + C3 + C4 products (acetate, ethanol, lactate, butyrate, butanol, and acetone) was calculated using previously described procedures.[23]

■ RESULTS

Ter-adhE1 Overexpression Pulls Carbon Flux toward Butanol. In our previous work, butanol production of *C. cellulovorans* was enhanced from 0.025 g/L to 1.64 g/L by overexpressing *adhE1* from *C. acetobutylicum* ATCC 824.[17] It illustrated that the heterologous butanol pathway can efficiently pull carbon flux from butyryl-CoA to butanol. Unfortunately, there was a considerable amount of residual sugars in the broth, which suggests that the carbon flow from sugar to butyrate and butanol can be further improved.

We investigated the CoA-dependent (carbon chain elongation) pathway and identified that *bcd* encodes a reversible and bifurcating butyryl-CoA dehydrogenase, that needs both NADH and oxidized Ferredoxin to reduce crotonyl-CoA to butyryl-CoA.[28,39] In order to avoid the reversible action of *bcd* catalyzing butyryl-CoA to crotonyl-CoA, thereby reducing the efficiency of the CoA-dependent (carbon chain elongation) pathway toward butyryl-CoA, we tried to establish an irreversible trans-enoyl-CoA reductase (Ter) from *Treponema denticola* to replace the native *bcd*.[28,29] However, we did not succeed in *bcd* inactivation or deletion after several attempts using either TargeTron or the CRISPR/nCas9 system. Therefore, the *T. denticola* Ter was overexpressed to pull carbon flux to butyryl-CoA as much as possible.

To explore the effect of Ter overexpression on carbon flux from crotonyl-CoA to butyryl-CoA, we measured the enzyme activity of both the strictly NADH dependent Ter as well as the $NADH^+$ oxidized Ferredoxin dependent (Bcd) crotonyl-CoA reductase activities (Figure 2A). Four different strains

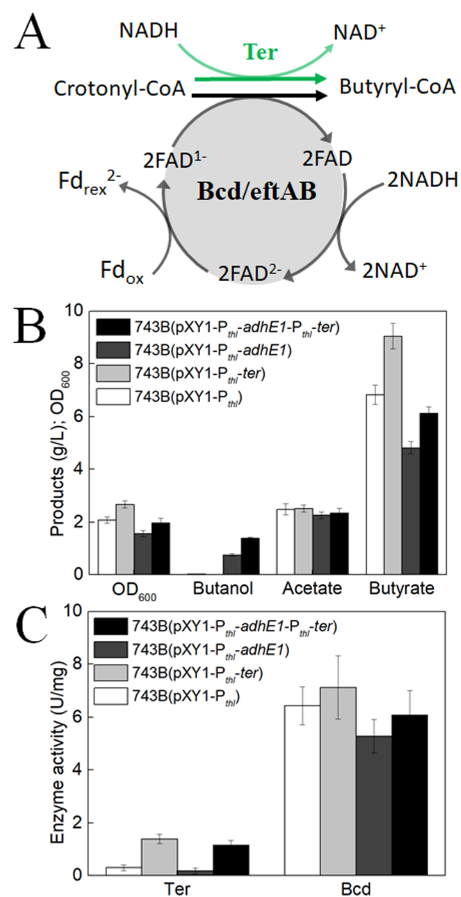

Figure 2. NADH oxidation assay method to measure Ter and Bcd activity for crotonyl-CoA. (A) Bcd needs both NADH and oxidized ferredoxin to reduce crotonyl-CoA to butyryl-CoA, while Ter only needs NADH. (B) Phenotype comparison of four different strains in 500 mL bioreactor containing 400 mL Clocel-medium with 50 g/L glucose as sole carbon source. (C) Ter and Bcd activity in different strains. Since there was no *ter* gene in 743B(pXY1-P_{thl}) and 743B(pXY1-P_{thl}-adhE1), Ter activity is almost zero in them at 48 h.

were cultured in Clocel medium with glucose as sole carbon source in 500 mL bioreactors with pH control at 7 and were sampled respectively at 48 h for enzyme activity measurement using a NADH oxidation assay method (Figure 2B and 2C). We found that in 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), the enzyme activity of Ter at 48 h was 19.1% of the Bcd. Given that one turnover of Bcd consumes almost 2 NADH, the flux ratio of Ter and Bcd is 1:2.62 at 48 h in 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter). That is, the overexpression of Ter increased the flux by 38.2% from crotonyl-CoA to butyryl-CoA at 48 h. As for 743B

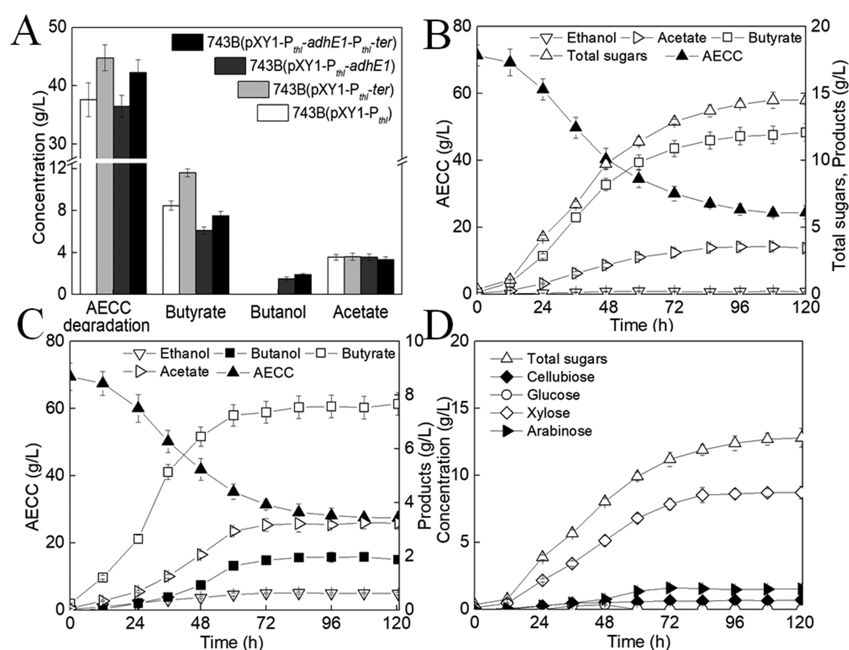

Figure 3. Ter-adhE1 overexpression to pull carbon flux toward butanol. (A) Effects of Ter overexpression on wild type and 743B(pXY1-P_{thl}-adhE1). (B) Fermentation profile of 743B(pXY1-P_{thl}-ter) with AECC as sole carbon source in a 3L bioreactor. (C and D) Fermentation profile of 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) with AECC as sole carbon source in a 3 L bioreactor.

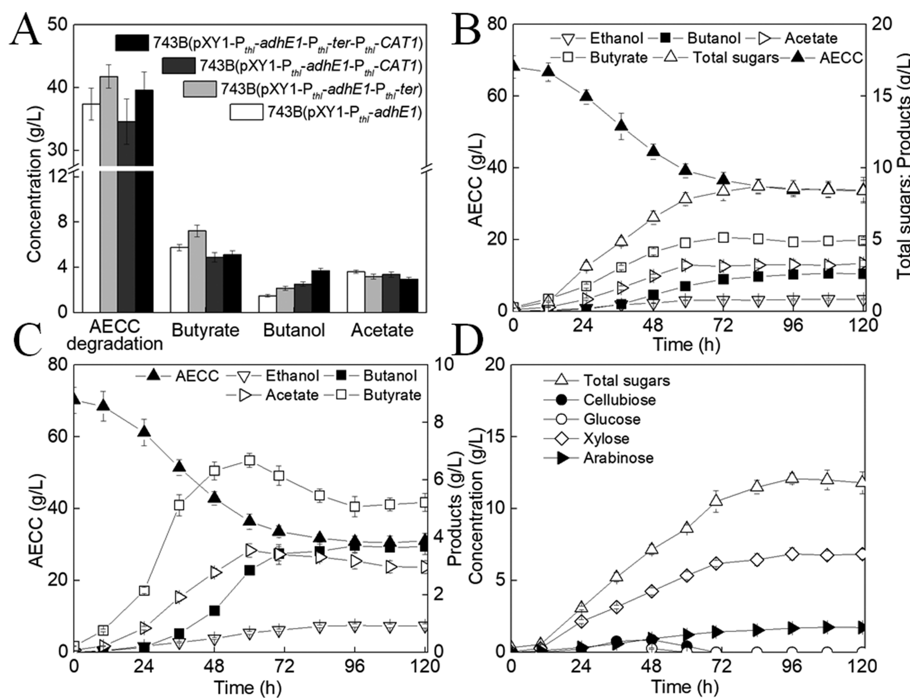

Figure 4. CAT1 overexpression to push butyrate reassimilation. (A) Effects of CAT1 overexpression on 743B(pXY1-P_{thl}-adhE1) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter). (B) Fermentation profile of 743B(pXY1-P_{thl}-adhE1-P_{thl}-CAT1) with AECC as sole carbon source in a 3L bioreactor. (C and D) Fermentation profile of 743B(pXY1 -P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1) with AECC as sole carbon source in a 3 L bioreactor.

(pXY1-P_{thl}-ter), the overexpression of Ter increased the flux by 39% at 48 h, and butyrate production of 743B (pXY1-P_{thl}-ter) is 32.5% higher than that of 743B (pXY1-P_{thl}) in the first 48 h (Figure 2B).

We then compared the effect of Ter overexpression on both the wild type and the strain 743B(pXY1-P_{thl}-adhE1) in the 500 mL bioreactor with AECC as sole carbon source (Figure 3A). We found that butyrate production by 743B(pXY1-P_{thl}-ter) increased by 36.8% compared to the wild type, and the AECC consumption increased by 7.2 g/L. The strain 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) showed a 26.3% higher production of butanol compared to 743B(pXY-P_{thl}-adhE1), which indicated that extra carbon flow is successfully pulled by Ter overexpression. Besides, 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) produced 7.51 g/L butyrate, which is enhanced by 22.7% compared to 743B-(pXY1-P_{thl}-adhE1) (Figure 3A).

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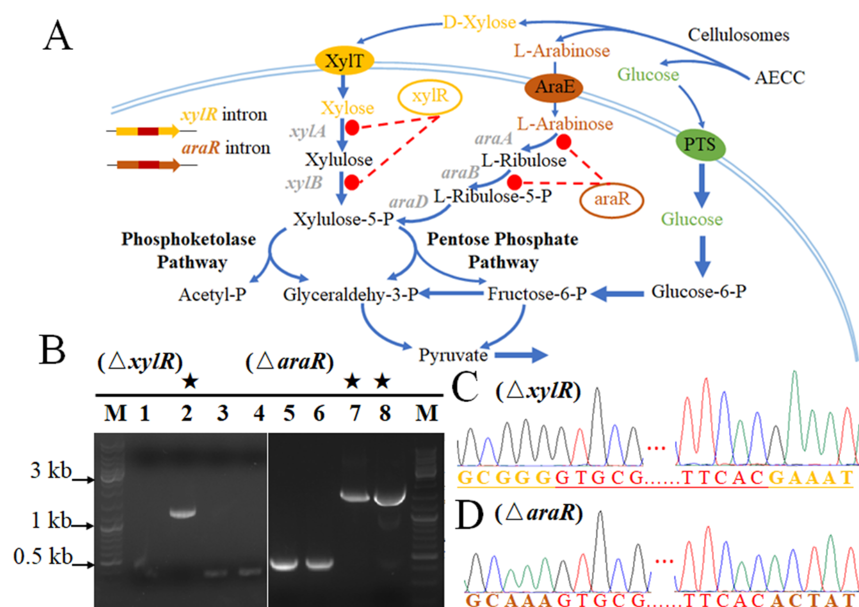

Figure 5. Confirmation of *xylR* and *araR* inactivation in *C. cellulovorans* by TageTron. (A) Function of xylR and araR in xylose metabolism. (B) Positive transformant screening by colony PCR. Lane 2, positive transformants for *xylR* knockout; Lanes 7 and 8, positive transformants for araR knockout; Lanes 3, 4, 5, and 6, negative transformants; and Lane 1, blank control. (C and D) DNA sequencing confirmed the correct insertion of the designed intron (bases in red bond) at the right locus. AraE, arabinose-proton symporter; *araA*, L-arabinose isomerase; *araB*, ribulokinase; *araD*, L-ribulose-5-phosphate 4-epimerase; araR, arabinose utilization negative regulator; XlyT, xylose proton-symporter; *xylA*, xylose isomerase; *xylB*, xylulokinase; xylR, xylose utilization negative regulator; PTS, phosphotransferase system.

Afterward, we investigated the fermentation progress of 743B(pXY1-P_{thl}-ter) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) in a 3 L bioreactor. We found that 743B(pXY1-P_{thl}-ter) decomposed 46.1 g/L AECC at 96 h and produced 11.8 g/L butyrate, with 14.2 g/L total sugars accumulation in the broth (Figure 3B). As for 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), it consumed 41.3 g/L AECC at 96 h and produced 1.96 g/L butanol (Figure 3C and 3D). The butanol titer is 78.4-fold of that by 743B wild type. However, it is worth noting that there are still 7.58 g/L of residual butyric acid in the broth of 743B (pXY1-P_{thl}-adhE1-P_{thl}-ter) (Figure 3C), which could be reassimilated to produce more butanol.

CAT1 Overexpression to Push Butyrate Reassimilation. We have previously observed the phenomenon of butyric acid reassimilation in the late stage of fermentation,[17,35,40] suggesting the presence of a reversible PTB-BK (phosphotransacetylase and butyrate kinase) channel in *C. cellulovorans*.[41,42] In order to reassimilate residual butyrate in the broth, we intend to reinforce or construct a butyrate reassimilated route uncoupled with acetone production. A butyryl-CoA–acetate CoA transferase (CAT1, encoded by *CTK_C06520*) from *C. tyrobutyricum* DSM 2637 has been demonstrated to directly catalyze butyryl-CoA and acetic acid to acetyl-CoA and butyric acid, without acetoacetate and acetone production.[30,31] We hypothesized that CAT1 could help promote the butyrate reassimilation of the PTB-BK channel or work reversibly to catalyze butyrate to butyryl-CoA in *C. cellulovorans*. Therefore, we attempted to overexpress CAT1 to construct alternative routes to push the carbon flux from butyric acid back to butyryl-CoA, which could then be pulled by Ter and AdhE1 toward butanol synthesis.

The effect of CAT1 overexpression on wild type, 743B-(pXY1-P_{thl}-adhE1), and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) was investigated in a 500 mL bioreactor with AECC as sole carbon source. We found that 743B(pXY1-P_{thl}-CAT1) decomposed 42.6 g/L AECC and produced 10.7 g/L butyrate, 13.3% and 26.2%, respectively, higher than those obtained by wild type (data not shown). However, data shows that butanol production by 743B(pXY1-P_{thl}-adhE1-P_{thl}-CAT1) increased by 69.6% compared to 743B(pXY1-P_{thl}-adhE1), and the residual butyrate decreased by 0.87 g/L (Figure 4A). The strain 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1), increased by 47.8% the butanol production compared to 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), and its residual butyrate decreased by 2.1 g/L (Figure 4A). Besides, neither acetoacetate nor acetone was detected in the fermentation broth. These results support our hypothesis to some extent.

Interestingly, CAT1 overexpression resulted in 6.94% and 7.36% decrease of acetic acid production in 743B(pXY1-P_{thl}-adhE1-P_{thl}-CAT1) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1), respectively, compared to those obtained by 743B-(pXY1-P_{thl}-adhE1) and 743B(pXY1 -P_{thl}-adhE1-P_{thl}-ter) (Figure 4A). It seems that CAT1 converted acetate to acetyl-CoA but did not work reversely to enhance acetate production.

We then explored the effect of CAT1 overexpression on the PTB-BK channel (Figure S1A and S1B). We first investigated the effects of 1 g/L of butyric acid addition on butanol production and *ptb/bk* (encoded by *Clocel_3675/Clocel_3674*) transcriptional levels in 743B (pXY1-P_{thl}-adhE1). We found that the addition of butyric acid promoted the production of butanol by 19.2% in the first 48 h. Meanwhile, the transcriptional level of *ptb* and *bk* increased by 136% and 109%, respectively, compared to that without butyrate addition at 48 h. In parallel, we examined the effect of CAT1 overexpression on *ptb/bk* expression. It is found that the transcriptional level of *ptb* and *bk* increased by 214% and 253%, respectively, in 743B (pXY1-P_{thl}-adhE1-P_{thl}-CAT1), compared to those in 743B (pXY1-P_{thl}-adhE1) at 48 h. Those similar results suggest that the effect of CAT1 overexpression on the PTB-BK channel may be achieved by

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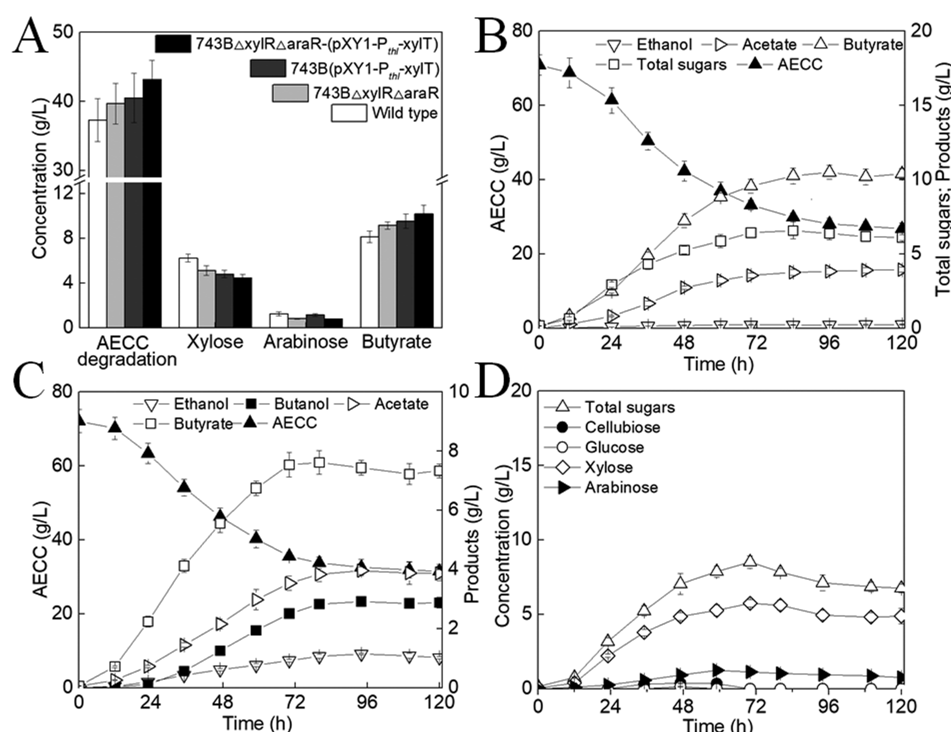

Figure 6. Xylose metabolic engineering to further improve AECC degradation and xylose utilization. (A) Fermentation phenotype comparison of 743B wild type, 743B△xylR△araR, 743B(pXY1-P_{thl}-xylT), and 743B△xylR△araR-(pXY1-P_{thl}-xylT) in a 500 mL bioreactor. (B) Fermentation profile of 743B△xylR△araR-(pXY1-P_{thl}-xylT) with AECC as sole carbon source in a 3L bioreactor. (C and D) Fermentation profile of 743B△xylR△araR-(pXY1-P_{thl}-adhE1-P_{thl}-xylT) with AECC as sole carbon source in a 3L bioreactor.

increasing butyric acid production, because the direct result of CAT1 overexpression is butyric acid enhancement. In addition, we designed experiments to knock out *bk* or *ptb* but failed to obtain mutant strains until now, which prevented us from further studying how CAT1 overexpression affects the PTB-BK channel.

We investigated the metabolic profile of 743B(pXY1-P_{thl}-adhE1-P_{thl}-CAT1) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1) in a 3 L bioreactor. 743B(pXY1-P_{thl}-adhE1-P_{thl}-CAT1) degraded 34.3 g/L AECC at 96 h and produced 2.57 g/L butanol (Figure 4B). The strain 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1) decomposed 39.4 g/L of AECC at 96 h and produced 3.71 g/L butanol, which is 89.3% more than that obtained by 743B (pXY1 -P_{thl}-adhE1-P_{thl}-ter) (Figure 4C). It should be noted that at the end of the fermentation (at 120 h), 2.97 g/L acetic acid and 5.22 g/L butyric acid were residual in broth of 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1), which is 90.1% and 68.0% of those residual in broth of 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) (Figure 4C and 3C). All above implied that CAT1 overexpression successfully promoted acids reassimilation and butanol production. However, there are still 6.85 g/L of xylose and 1.68 g/L of arabinose in the broth of 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1) (Figure 4D), which suggests an inefficient xylose utilization of *C. cellulovorans.*

Xylose Metabolic Engineering to Further Drive Butanol Synthesis. Similar to most clostridia, *C. cellulovorans* cannot utilize pentoses efficiently due to the carbon catabolite repression (CCR),[43,44] which might reduce the butanol yield from lignocellulosic biomass, as xylose is the second most abundant sugar in the lignocellulosic hydrolysates.[45] Besides, carbon, ATP, and reducing power that derived from efficient xylose utilization are important driving forces for the CoA-dependent (carbon chain elongation) pathway and the butanol synthesis pathway.[23] Therefore, xylose metabolic engineering was performed in *C. cellulovorans* to further enhance butanol production.

To accomplish this goal, xylR (*Clocel_0594*, encoding a xylose utilization negative regulator) and araR (*Clocel_1253*, encoding an arabinose utilization negative regulator) were inactivated by TargeTron, and then xylT (*CA_C1345*, encoding a D-xylose proton-symporter from *C. acetobutylicum* ATCC 824) was overexpressed,[32] as shown in Figure 5. A similar strategy has been previously shown effective by Xiao et al. in *C. beijerinckii* NCIMB 8052.[46]

The fermentation phenotypes of 743B wild type, 743B△xylR△araR, 743B(pXY1-P_{thl}-xylT), and 743B△xylR△araR-(pXY1-P_{thl}-xylT) in a 500 mL bioreactor with AECC as sole carbon source were compared with each other (Figure 6A). We found that residual xylose by 743B△xylR△araR decreased by 18% compared to wild type, but its AECC consumption increased by 2.4 g/L. XylT overexpression in 743B△xylR△araR further decreased residual xylose to 4.45 g/L in broth. Besides, xylose metabolic engineering also improved butyrate production from 8.13 g/L to 10.2 g/L, indicating that carbon flow was driven toward CoA-dependent (carbon chain elongation) pathway.

Then the fermentation profile of 743B△xylR△araR-(pXY1-P_{thl}-xylT) and 743B△xylR△araR-(pXY1-P_{thl}-adhE1-P_{thl}-xylT) was investigated in a 3 L bioreactor. It was found that 743B△xylR△araR-(pXY1-P_{thl}-xylT) decomposed 42.8 g/L AECC at 96 h and produced 10.5 g/L butyrate, with 4.62 g/L of residual xylose in the broth (Figure 6B). The strain 743B△xylR△araR-(pXY1-P_{thl}-adhE1-P_{thl}-xylT) consumed 39.5 g/L AECC at 94 h and produced 2.92 g/L butanol, which is 78% higher than that by 743B(pXY1-P_{thl}-adhE1) (Figure 6C and 6D). However, we found that 7.44 g/L of

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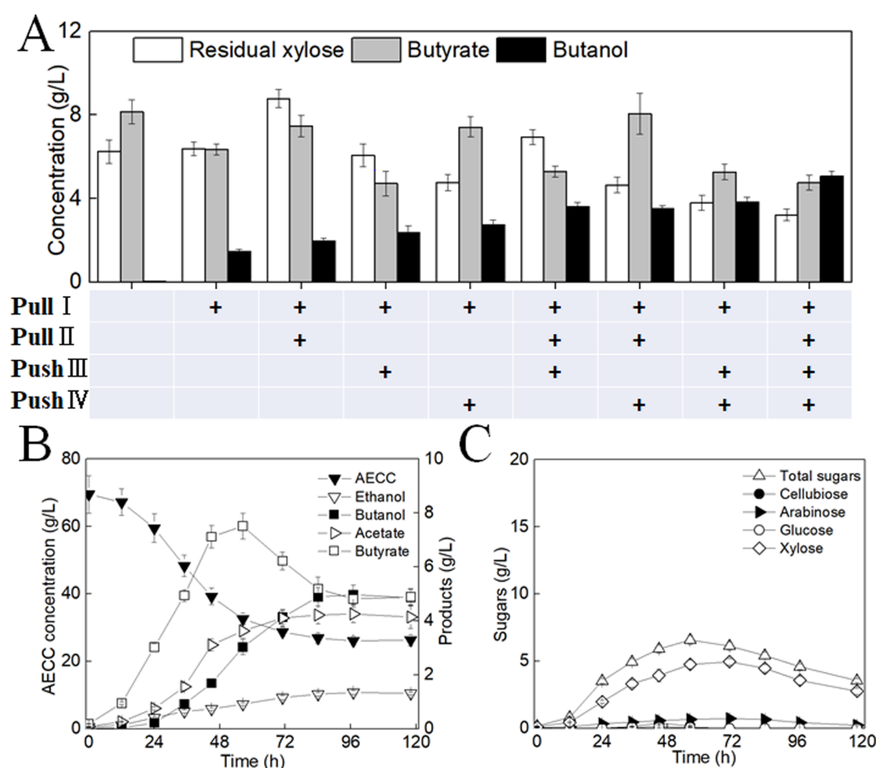

Figure 7. All push−pull modules assembly to maximize butanol production. (A) Effects of modules assembly on fermentation profile in residual xylose, as well as butanol and butyrate production. (B and C) Fermentation profile of 743B△xylR△araR-(pXY1-P_{thl}-adhE1-P_{thl}-ter-P_{thl}-CAT1-P_{thl}-xylT) with AECC as sole carbon source in a 3 L bioreactor.

residual butyrate was accumulated in the broth of 743B△xylR△araR-(pXY1-P_{thl}-adhE1-P_{thl}-xylT) (Figure 6C), which might be attributed to an enhanced flux from xylose metabolism.

Combination of the Push−Pull Modules Maximizes Butanol Production. We decided to include all the above-described push−pull modules in *C. cellulovorans* DSM 743B for a further improvement of *n*-butanol production. Comparing the metabolic profiles among mutants with different modules combination (Figure 7A), we found that the assembly of four modules one by one in the strains has a stepwise promotion of the butanol titer from to 0.021 to 4.96 g/L (Figure 8). Simultaneously, the residual butyrate and xylose decreased gradually from 8.15 and 6.24 g/L to 4.76 and 3.22 g/L, respectively (Figure 7A). These results indicate that the metabolic engineering approaches based on push−pull strategies can effectively maximize the metabolic flux toward butanol synthesis.

Batch fermentation in a 3 L bioreactor was performed to investigate the metabolic profile of the strain 743B△xylR△araR-(pXY1P_{thl}-adhE1-P_{thl}-ter- P_{thl}-CAT1-P_{thl}-xylT) (Figure 7B and 7C). During the first 45 h, AECC degradation and butyrate and butanol production were substantially improved compared to those in Figures 3C, 4B, and 6B, while xylose and total sugars did not accumulate significantly, which indicates the positive effect of combining multiple modules. From 56.5 to 97 h, 2.7 g/L butyrate was reassimilated, and 1.19 g/L xylose was further consumed, which further promoted butanol production by 1.94 g/L. At 97 h, the strain 743B△xylR△araR-(P_{thl}-adhE1- P_{thl}-ter- P_{thl}-CAT1-P_{thl}-xylT) produced 4.96 g/L *n*-butanol and 1.35 g/L ethanol from 43.4 g/L AECC with 4.81 g/L of butyrate and 3.56 g/L of residual xylose in the broth (Figure 7B and 7C). Compared to wild type, the *n*-butanol production increased by 235-fold.

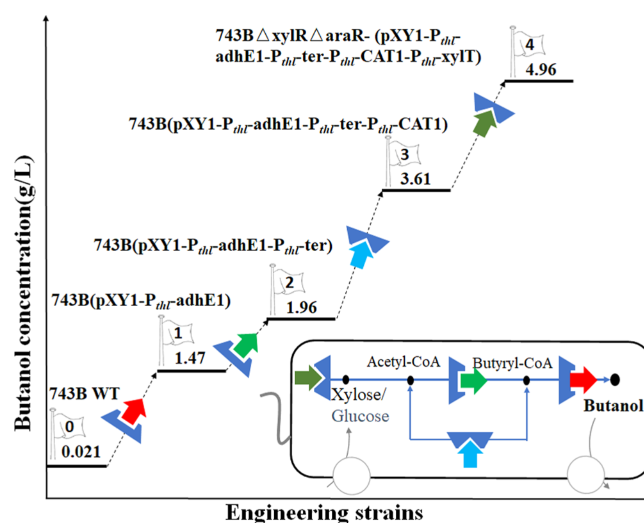

Figure 8. Modules assembly has a stepwise promotion to butanol titer. These pull−push modules worked like engines, providing driving force and pulling power toward butanol. The *n*-butanol production of strain with four modules achieved 4.96 g/L, increasing by over 235-fold compared to the wild type.

■ DISCUSSION

In this study, a metabolic engineering approach based on a push−pull strategy was developed to enhance cellulosic butanol production of *C. cellulovorans* by consolidated bioprocessing. After genetically modifying three different

Table 1. Comparison of (Iso)butanol Production with Recombinant Cellulolytic Clostridia by CBP

Strains	Genotype	Substrate	Titer (g/L)	Productivity (g/L/h)	References
C. cellulolyticum ATCC 35319	*+kivD-yqhD-alsS-ilvCD*	Crystalline cellulose	0.66[a]	0.0031	14
C. cellulolyticum ATCC 35319	*+ato-hbd-crt-bcd-adhE2*	Crystalline cellulose	0.12	0.00025	59
C. cellulolyticum ATCC 35319	*ΔSpo0A+kivD-alsS*	Crystalline cellulose	0.42[a]	ND	60
C. thermocellum DSM 1313	*Δhpt+ilvBNCD-kivD*	Crystalline cellulose	5.4[a]	0.072	15
C. thermocellum DSM 1313	*ΔhptΔClo1313Δldh+(Tt_thl-Tt_hbd, Tt_crt-St_ter-Ts_bad-Ts_bdh)*[b] *+Tt_thlM2-Tt_hbdM-St_terM*	Crystalline cellulose	0.357	0.00298	16
C. cellulovoran DSM 743B	*+adhE2*	Crystalline cellulose	1.42	0.0056	22
C. cellulovoran DSM 743B	*+adhE2*	Pretreated corn cob	3.36	0.028	40
C. cellulovoran DSM 743B	*+adhE2*	Crystalline cellulose	4.0	0.0128	21
C. cellulovoran DSM 743B	*(ΔSpo0A + adhE1-ctfAB-adc)*[c]	AECC	3.47	0.0413	17
C. cellulovoran DSM 743B	*ΔXylRΔaraR+ adhE1-ter-CAT1-xylT*	AECC	4.96	0.0511	This study

[a]Titer of isobutanol. [b]Genome integration. [c]Evolved strain; ND, not detected; +, overexpression; Δ, deficient or inactivation; AECC, alkali extracted corn cobs.

pathways to direct carbon flow toward butyryl-CoA, the *n*-butanol production finally reached 4.96 g/L (Figure 8), suggesting that *C. cellulovorans* has a great potential as CBP-enabling chassis strain. This strategy, compared to previous ones to produce *n*-butanol or isobutanol in a CBP with single recombinant clostridia using lignocellulosic biomasses, presents advantages in both titers and productivity (Table 1).

Our push−pull strategy includes several modules to maximize the carbon flux toward butanol synthesis. First, an alcohol aldehyde dehydrogenase gene *adhE1* from *Clostridium acetobutylicum* ATCC 824 was introduced to construct a complete butanol synthesis pathway in *C. cellulovorans* to produce butanol.[17] Interestingly, it has been demonstrated that purified AdhE1 has a strong NADH dependent butyraldehyde dehydrogenase activity while having very low butanol dehydrogenase activity.[47,48] Therefore, the butanol dehydrogenase activity we measured in 743B(pXY1-P_{thl}-adhE1) might come mainly from the strain itself (Figure S2B). The effect of 0.5 g/L of butyraldehyde on butanol dehydrogenase activity confirms that (Figure S2). We found that, with 0.5 g/L butyraldehyde addition, the wild strain produced 0.118 g/L of butanol at 48 h, which is 6.41-fold of that obtained without addition (Figure S2A). Besides, butyraldehyde addition results in butanol dehydrogenase activity increasing by 2.68-fold (Figure S2B), suggesting that the butanol dehydrogenase-encoding genes may be up-expressed in response to butyraldehyde. It explained to some extent why *n*-butanol is produced when adhE1 is overexpressed. Anyway, adhE1 introduction successfully pulled carbon flux from butyryl-CoA to butanol.

Afterward, we overexpressed an irreversible butyryl-CoA dehydrogenase (Ter) facilitating the conversion of acetyl-CoA to butyryl-CoA, which provides more substrate for the butanol synthesis by AdhE1 (Figure 2A and 3). Enzyme activity of Ter and Bcd at 48 h in 743B(pXY1-P_{thl}-ter) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter) provided a better understanding of the role of each enzyme in this flux (Figure 2). However, it should be noted that Bcd is not very stable, and the measured enzyme activity is usually lower than the actual value.[28,49,50] Therefore, the actual flux increase caused by Ter overexpression is less than 39% and 38.2% at 48 h in 743B(pXY1-P_{thl}-ter) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), respectively. Generally, we believe that Bcd plays a major role in743B(pXY1-P_{thl}-ter) and 743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), which explains why Ter overexpression caused only 32.5% of increase in butyric acid in 743B (pXY1-P_{thl}-ter) and 35.8% of enhancement in butyrate/butanol production in743B(pXY1-P_{thl}-adhE1-P_{thl}-ter), respectively, in the first 48 h (Figure 2B).

However, we have not obtained a mutant with deficient native *bcd* gene (encoding a reversible butyryl-CoA dehydrogenase) here, which prevented us from further analyzing the contribution of Bcd in the flux of butyrate/butanol production. There were also no studies reporting that *bcd* gene in *C. acetobutylicum* ATCC 824 has been successfully inactivated. Based on the hypothesis that butyryl-CoA dehydrogenase is essential for *C. cellulovorans*, TargeTron or the CRISPR/nCas9 system with Ter overexpression in the same plasmids system were attempted but were not successful until now. It might be partly attributed to the inefficiency of the two genetic tools. Inactivation efficiency of TargeTron is sometimes gene specific (or dependent).[51,52] As for CRISPR/nCas9, almost no transformant was observed for *bcd* in-frame deletion in our study (data not shown), which might result from inefficient homologous recombination capacity and low plasmids transformation efficiency in *C. cellulovorans*.[23] Recently, a CRISPR-$Cas9^{D10A}$-assisted base editing system was developed,[53] which can create a missense mutation or null mutations in a gene. It is as precise as Cas9-mediated genome editing but does not have to result in DNA double strands break (DSB), which is usually fatal to *Clostridium*. It might be introduced to inactivate *bcd* here. In preparing the paper, Strecker et al. developed a RNA-guided DNA insertion method with CRISPR-associated transposases in *Escherichia coli*,[54] which is also expected to be introduced to *C. cellulovorans* and facilitate programmable gene interruption or insertion based on nonhomologous recombination. Admittedly, lack of efficient genetic tools development is still an obstacle for complicated genetical modification in *C. cellulovorans*.[10,55]

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The main driving force for butanol production in the work comes from acetate/butyrate reassimilation and xylose metabolic engineering. In solventogenic clostridia, acids reassimilation reinforcement by overexpressing *ctfAB* is a routine strategy to push carbon flux toward butanol synthesis,[56,57] which we applied in cellulolytic clostridia. We previously expressed the *ctfAB* gene encoding an acetate/butyrate-acetoacetate COA-transferase from *C. acetobutylicum* ATCC 824 in *C. cellulovorans*.[17] Unfortunately, an *adc* gene (encoding an acetoacetate decarboxylase) must be overexpressed synchronously to ensure that the unstable intermediate product acetoacetate generated by butyrate reassimilation can be metabolized to the end-product acetone.[17,24] The *ctfAB-adc* operon overexpression resulted in the accumulation of the undesired byproduct (acetone) in the broth. Besides, we failed to achieve butyrate reassimilation by introducing bifunctional BK-I encoded by bukI (*CA_c3075*) from *C. acetobutylicum*.[17] Interestingly, CAT1 overexpression in the study promoted butyrate reassimilation and subsequent butanol production (Figure 4). We confirmed that CAT1 overexpression indeed affects the PTB-BK channel (in transcription level of *ptb/bk*) (Figure S1A and S1B). We speculated that CAT1 overexpression rebalanced butyric acid and butyryl-CoA by regulating the reversible PTB-BK channel. It is presumably because CAT1 overexpression produced butyrate using an alternative pathway, which stimulated a reversed reaction from butyrate to butyryl-CoA via the PTB-BK channel in *C. cellulovorans*.[41,42]However, we have not obtained a *buk* (*Clocel_3674*, encoding butyrate kinase) or *ptb* (*Clocel_3675*, encoding phosphotransacetylase) deficient strain; it remains unclear how CAT1 overexpression affects the direction and carbon flux of the PTB-BK channel. It has been reported that *cat1*(*CTK_C06520*) is the only gene responsible for butyrate biosynthesis in *C. tyrobutyricum*.[30,31] Inactivation of *cat1* using a mobile group II intron was not successful because of its essential role in ATP generation.[30] Our study represents an attempt of heterogeneous expression, providing a different perspective of *cat1* function study in other species.

Regarding xylose metabolic engineering, we here improved xylose assimilation, which provided extra carbon, reducing power (NADPH), and energy (ATP) for CoA-dependent (carbon chain elongation) pathway, acids reassimilation, and butanol synthesis.[23,32,46] Moreover, the decrease in residual sugar concentration weakened the feedback inhibition to cellulase expression, which explains why the degradation of AECC was promoted to some extent after xylose metabolic engineering (Figure 6). In fact, the efficient degradation of AECC, the sole carbon source, is the fundamental driving force for all metabolism in our scenario.[23]

The engineered strain here has demonstrated the advantages of the push−pull strategy in metabolic pathway design (Table 1). However, in the work, we mainly focused on carbon flux direction in *C. cellulovorans*. The improvements in reducing power (NADPH or NADH) and bioenergy (such as ATP) have not been exploited yet. A potential way to further increase butanol production in the generated strains would be to reduce the production of lactic acid and hydrogen,[23] which drains NADPH away from the product of interest. In addition, further optimization in the future could come from the use of dynamic or genome-scale metabolic network model-driven analysis,[58] so that the driving forces or pulling power can be rationally designed and regulated for higher efficiency and robustness. Besides, the tolerance of butanol is critical for further optimization, because it determines the current upper limit of butanol production. We previously obtained an evolved strain with high butanol tolerance and proved that an enhanced butanol resistance can improve butanol production to some extent.[17] We anticipate that the use of evolved chassis, coupled with recent progress in genetic tools development[10,53,55] as well as rational metabolic engineering strategies, will enable the development of the next generation of *C. cellulovorans* strains.

■ CONCLUSIONS

C. cellulovorans DSM 743B is considered a potential chassis strain for *n*-butanol production by consolidated bioprocessing (CBP). However, its *n*-butanol production potential from lignocellulosic biomass has not been fully unexplored. Here, a metabolic engineering based on a push−pull strategy was developed to exploit cellulosic butanol production of *C. cellulovorans* from alkali-extracted corn cobs (AECC). The engineered strain finally produced 4.96 g/L of *n*-butanol, which is 236-fold of that obtained from the wild type. Thus, the engineered strain serves as a promising platform for butanol production from lignocellulose by CBP.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00331.

Supplementary Tables S1−S4, Supplementary Figures S1−S2, and Supplementary DNA sequence (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Mingjie Jin − *School of Environmental and Biological Engineering, Nanjing University of Science & Technology, Nanjing 210094, China;* orcid.org/0000-0002-9493-305X; Email: jinmingjie@njust.edu.cn

Sheng Yang − *Key Laboratory of Synthetic Biology, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology and Huzhou Center of Industrial Biotechnology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China;* orcid.org/0000-0003-3742-9989; Email: syang@sibs.ac.cn

Authors

Zhiqiang Wen − *School of Environmental and Biological Engineering, Nanjing University of Science & Technology, Nanjing 210094, China*

Rodrigo Ledesma-Amaro − *Department of Bioengineering, Imperial College London, London SW7 2AZ, U.K.*

Minrui Lu − *School of Environmental and Biological Engineering, Nanjing University of Science & Technology, Nanjing 210094, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00331

Author Contributions

Zhiqiang Wen and Minrui Lu performed the experiments, and analyzed the data; Sheng Yang and Mingjie Jin coordinated and supervised this study; Zhiqiang Wen, Mingjie Jin, Sheng Yang, and Rodrigo Ledesma-Amaro drafted and revised the manuscript.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Jiang, Y., Liu, J. L., Jiang, W. H., Yang, Y. L., and Yang, S. (2015) Current status and prospects of industrial bio-production of n-butanol in China. *Biotechnol. Adv. 33*, 1493−1501.

(2) Nguyen, N. P. T., Raynaud, C., Meynial-Salles, I., and Soucaille, P. (2018) Reviving the Weizmann process for commercial n-butanol production. *Nat. Commun. 9*, 9.

(3) Cho, C., Jang, Y.-S., Moon, H. G., Lee, J., and Lee, S. Y. (2015) Metabolic engineering of clostridia for the production of chemicals. *Biofuels, Bioprod. Biorefin. 9*, 211−225.

(4) Fast, A. G., Schmidt, E. D., Jones, S. W., and Tracy, B. P. (2015) Acetogenic mixotrophy: novel options for yield improvement in biofuels and biochemicals production. *Curr. Opin. Biotechnol. 33*, 60−72.

(5) Ng, T., Weimer, P., and Zeikus, J. (1977) Cellulolytic and physiological properties of *Clostridium thermocellum*. *Arch. Microbiol. 114*, 1−7.

(6) Petitdemange, E., Caillet, F., Giallo, J., and Gaudin, C. (1984) *Clostridium cellulolyticum* sp. nov., a cellulolytic, mesophilic: species from decayed grass. *Int. J. Syst. Bacteriol. 34*, 155−159.

(7) Sleat, R., Mah, R. A., and Robinson, R. (1984) Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. *Appl. Environ. Microbiol. 48*, 88−93.

(8) Olson, D. G., McBride, J. E., Shaw, A. J., and Lynd, L. R. (2012) Recent progress in consolidated bioprocessing. *Curr. Opin. Biotechnol. 23*, 396−405.

(9) Lynd, L. R., van Zyl, W. H., McBride, J. E., and Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: an update. *Curr. Opin. Biotechnol. 16*, 577−583.

(10) Joseph, R. C., Kim, N. M., and Sandoval, N. R. (2018) Recent Developments of the Synthetic Biology Toolkit for *Clostridium*. *Front. Microbiol. 9*, 9.

(11) Minton, N. P., Ehsaan, M., Humphreys, C. M., Little, G. T., Baker, J., Henstra, A. M., Liew, F., Kelly, M. L., Sheng, L., Schwarz, K., and Zhang, Y. (2016) A roadmap for gene system development in *Clostridium*. *Anaerobe 41*, 104−112.

(12) Pyne, M. E., Bruder, M., Moo-Young, M., Chung, D. A., and Chou, C. P. (2014) Technical guide for genetic advancement of underdeveloped and intractable *Clostridium*. *Biotechnol. Adv. 32*, 623−641.

(13) Gaida, S. M., Liedtke, A., Jentges, A. H., Engels, B., and Jennewein, S. (2016) Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose. *Microb. Cell Fact. 15*, 6.

(14) Higashide, W., Li, Y., Yang, Y., and Liao, J. C. (2011) Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl. Environ. Microbiol. 77*, 2727−2733.

(15) Lin, P. P., Mi, L., Morioka, A. H., Yoshino, K. M., Konishi, S., Xu, S. C., Papanek, B. A., Riley, L. A., Guss, A. M., and Liao, J. C. (2015) Consolidated bioprocessing of cellulose to isobutanol using *Clostridium thermocellum*. *Metab. Eng. 31*, 44−52.

(16) Tian, L., Conway, P. M., Cervenka, N. D., Cui, J., Maloney, M., Olson, D. G., and Lynd, L. R. (2019) Metabolic engineering of *Clostridium thermocellum* for n-butanol production from cellulose. *Biotechnol. Biofuels 12*, 186.

(17) Wen, Z., Ledesma-Amaro, R., Lin, J., Jiang, Y., and Yang, S. (2019) Improved n-butanol production from *Clostridium cellulovorans* by integrated metabolic and evolutionary engineering. *Appl. Environ. Microbiol. 85*, 85.

(18) Shoseyov, O., and Doi, R. H. (1990) Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. *Proc. Natl. Acad. Sci. U. S. A. 87*, 2192−2195.

(19) Shoseyov, O., Takagi, M., Goldstein, M. A., and Doi, R. H. (1992) Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. *Proc. Natl. Acad. Sci. U. S. A. 89*, 3483−3487.

(20) Yang, X., Xu, M., and Yang, S. T. (2016) Restriction modification system analysis and development of in vivo methylation for the transformation of *Clostridium cellulovorans*. *Appl. Microbiol. Biotechnol. 100*, 2289−2299.

(21) Bao, T., Zhao, J., Li, J., Liu, X., and Yang, S.-T. (2019) n-Butanol and ethanol production from cellulose by *Clostridium cellulovorans* overexpressing heterologous aldehyde/alcohol dehydrogenases. *Bioresour. Technol. 285*, 121316−121316.

(22) Yang, X., Xu, M., and Yang, S. T. (2015) Metabolic and process engineering of *Clostridium cellulovorans* for biofuel production from cellulose. *Metab. Eng. 32*, 39−48.

(23) Wen, Z., Minton, N. P., Zhang, Y., Li, Q., Liu, J., Jiang, Y., and Yang, S. (2017) Enhanced solvent production by metabolic engineering of a twin-clostridial consortium. *Metab. Eng. 39*, 38−48.

(24) Gong, F., Bao, G., Zhao, C., Zhang, Y., Li, Y., and Dong, H. (2016) Fermentation and genomic analysis of acetone-uncoupled butanol production by *Clostridium tetanomorphum*. *Appl. Microbiol. Biotechnol. 100*, 1523−1529.

(25) Lv, X., Xie, W., Lu, W., Guo, F., Gu, J., Yu, H., and Ye, L. (2014) Enhanced isoprene biosynthesis in Saccharomyces cerevisiae by engineering of the native acetyl-CoA and mevalonic acid pathways with a push-pull-restrain strategy. *J. Biotechnol. 186*, 128−136.

(26) Tai, M., and Stephanopoulos, G. (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metab. Eng. 15*, 1−9.

(27) Fang, M., Wang, T., Zhang, C., Bai, J., Zheng, X., Zhao, X., Lou, C., and Xing, X.-H. (2016) Intermediate-sensor assisted push-pull strategy and its application in heterologous deoxyviolacein production in *Escherichia coli*. *Metab. Eng. 33*, 41−51.

(28) Shen, C. R., Lan, E. I., Dekishima, Y., Baez, A., Cho, K. M., and Liao, J. C. (2011) Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol. 77*, 2905−2915.

(29) Dekishima, Y., Lan, E. I., Shen, C. R., Cho, K. M., and Liao, J. C. (2011) Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered Escherichia coli. *J. Am. Chem. Soc. 133*, 11399−11401.

(30) Lee, J., Jang, Y. S., Han, M. J., Kim, J. Y., and Lee, S. Y. (2016) Deciphering *Clostridium tyrobutyricum* metabolism based on the whole-genome sequence and proteome analyses. *mBio 7*, 7.

(31) Zhang, J. Z., Yu, L., Xu, M. M., Yang, S. T., Yan, Q. J., Lin, M., and Tang, I. C. (2017) Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production from sugarcane juice. *Appl. Microbiol. Biotechnol. 101*, 4327−4337.

(32) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., and Yang, S. (2011) Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Appl. Environ. Microbiol. 77*, 7886−7895.

(33) Zhang, N., Shao, L., Jiang, Y., Gu, Y., Li, Q., Liu, J., Jiang, W., and Yang, S. (2015) I-SceI-mediated scarless gene modification via allelic exchange in *Clostridium*. *J. Microbiol. Methods 108*, 49−60.

(34) Wu, Y., Yang, Y., Ren, C., Yang, C., Yang, S., Gu, Y., and Jiang, W. (2015) Molecular modulation of pleiotropic regulator CcpA for glucose and xylose coutilization by solvent-producing *Clostridium acetobutylicum*. *Metab. Eng. 28*, 169−179.

(35) Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., and Cen, P. (2014) Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and *Clostridium cellulovorans*. *Microb. Cell Fact. 13*, 1−11.

https://dx.doi.org/10.1021/acssynbio.9b00331
ACS Synth. Biol. XXXX, XXX, XXX−XXX

(36) Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., and Thauer, R. K. (2008) Coupled ferredoxin and crotonyl coenzyme a (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. *J. Bacteriol. 190*, 843−850.
(37) Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., and Cen, P. (2014) A novel strategy for sequential co-culture of *Clostridium thermocellum* and *Clostridium beijerinckii* to produce solvents from alkali extracted corn cobs. *Process Biochem. 49*, 1941−1949.
(38) Chen, S., Xu, Z., Li, X., Yu, J., Cai, M., and Jin, M. (2018) Integrated bioethanol production from mixtures of corn and corn stover. *Bioresour. Technol. 258*, 18−25.
(39) Buckel, W., and Thauer, R. K. (2018) Flavin-based electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiration with protons (Ech) or NAD+ (Rnf) as electron acceptors: a historical review. *Front. Microbiol. 9*, 401.
(40) Ou, J., Xu, N., Ernst, P., Ma, C., Bush, M., Goh, K., Zhao, J., Zhou, L., Yang, S.-T., and Liu, X. (2017) Process engineering of cellulosic n-butanol production from corn-based biomass using *Clostridium cellulovorans*. *Process Biochem. 62*, 144−150.
(41) 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 do, Y., and Lee, S. Y. (2012) Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. *mBio 3*, 3.
(42) Lehmann, D., Radomski, N., and Luetke-Eversloh, T. (2012) New insights into the butyric acid metabolism of *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol. 96*, 1325−1339.
(43) Mitchell, W. J. (2016) Sugar uptake by the solventogenic clostridia. *World J. Microbiol. Biotechnol. 32*, 32.
(44) Bruder, M., Moo-Young, M., Chung, D. A., and Chou, C. P. (2015) Elimination of carbon catabolite repression in *Clostridium acetobutylicum*-a journey toward simultaneous use of xylose and glucose. *Appl. Microbiol. Biotechnol. 99*, 7579−7588.
(45) Jin, M., Slininger, P. J., Dien, B. S., Waghmode, S., Moser, B. R., Orjuela, A., Sousal, L. d. C., and Balan, V. (2015) Microbial lipid-based lignocellulosic biorefinery: feasibility and challenges. *Trends Biotechnol. 33*, 43−54.
(46) Xiao, H., Li, Z. L., Jiang, Y., Yang, Y. L., Jiang, W. H., Gu, Y., and Yang, S. (2012) Metabolic engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. *Metab. Eng. 14*, 569−578.
(47) Yoo, M., Bestel-Corre, G., Croux, C., Riviere, A., Meynial-Salles, I., and Soucaille, P. (2015) A quantitative system-scale characterization of the metabolism of *Clostridium acetobutylicum*. *mBio 6*, e01808−01815.
(48) Yoo, M., Croux, C., Meynial-Salles, I., and Soucaille, P. (2016) Elucidation of the roles of adhE1 and adhE2 in the primary metabolism of *Clostridium acetobutylicum* by combining in-frame gene deletion and a quantitative system-scale approach. *Biotechnol. Biofuels 9*, 92.
(49) Boynton, Z. L., Bennet, G. N., and Rudolph, F. B. (1996) Cloning, sequencing, and expression of clustered genes encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol. 178*, 3015.
(50) Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., and Liao, J. C. (2007) Metabolic Engineering of *Escherichia coli* for 1-Butanol Production. *Metab. Eng. 10*, 305−311.
(51) Heap, J. T., Pennington, O. J., Cartman, S. T., Carter, G. P., and Minton, N. P. (2007) The ClosTron: A universal gene knock-out system for the genus *Clostridium*. *J. Microbiol. Methods 70*, 452−464.
(52) Enyeart, P. J., Mohr, G., Ellington, A. D., and Lambowitz, A. M. (2014) Biotechnological applications of mobile group II introns and their reverse transcriptases: gene targeting, RNA-seq, and non-coding RNA analysis. *Mobile DNA 5*, 5.
(53) Li, Q., Seys, F. M., Minton, N. P., Yang, J., Jiang, Y., Jiang, W., and Yang, S. (2019) CRISPR-Cas9(D10A) nickase-assisted base editing in the solvent producer *Clostridium beijerinckii*. *Biotechnol. Bioeng. 116*, 1475−1483.
(54) Strecker, J., and Ladha, A. (2019) RNA-guided DNA insertion with CRISPR-associated transposases. *Science 365*, 48−53.
(55) McAllister, K. N., and Sorg, J. A. (2019) CRISPR genome editing systems in the genus Clostridium: a timely advancement. *J. Bacteriol. 201*, e00219−19.
(56) Yu, L., Zhao, J., Xu, M., Dong, J., Varghese, S., Yu, M., Tang, I. C., and Yang, S.-T. (2015) Metabolic engineering of Clostridium tyrobutyricum for n-butanol production: effects of CoA transferase. *Appl. Microbiol. Biotechnol. 99*, 4917−4930.
(57) Jang, Y. S., Lee, J. Y., Lee, J. M., and Lee, S. Y. (2009) Metabolic engineering of *Clostridium acetobutylicum* M5 for butanol-ethanol production by complementation of *adhE1* and *ctfAB* genes. *New Biotechnol. 25*, S273−S274.
(58) Liao, C., Seo, S. O., Celik, V., Liu, H., Kong, W., Wang, Y., Blaschek, H., Jin, Y. S., and Lu, T. (2015) Integrated, systems metabolic picture of acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum*. *Proc. Natl. Acad. Sci. U. S. A. 112*, 8505−8510.
(59) Gaida, S. M., Liedtke, A., Jentges, A. H. W., Engels, B., and Jennewein, S. (2016) Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose. *Microb. Cell Fact. 15*, 1.
(60) Li, Y., Xu, T., Tschaplinski, T. J., Engle, N. L., Yang, Y., Graham, D. E., He, Z., and Zhou, J. (2014) Improvement of cellulose catabolism in *Clostridium cellulolyticum* by sporulation abolishment and carbon alleviation. *Biotechnol. Biofuels 7*, 25.