AEM Accepted Manuscript Posted Online 18 January 2019
Appl. Environ. Microbiol. doi:10.1128/AEM.02560-18
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Improved n-butanol production from *Clostridium cellulovorans* by integrated metabolic and evolutionary engineering

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Author Contributions

Zhiqiang Wen, Yu Jiang and Sheng Yang performed the studies and drafted the manuscript, Rodrigo Ledesma-Amaro and Jianping Lin drafted and revised the manuscript.

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Abstract

Clostridium cellulovorans DSM 743B offers potential as a chassis strain for biomass refining by consolidated bioprocessing (CBP). However, its n-butanol production from lignocellulosic biomass has yet to be demonstrated. This study demonstrates the construction of a CoA-dependent acetone-butanol-ethanol (ABE) pathway in *C. cellulovorans* by introducing genes of *adhE1* and *ctfA-ctfB-adc* from *C. acetobutylicum* ATCC 824, which enabled it to produce n-butanol using the abundant and low-cost agricultural waste of alkali-extracted, deshelled corn cobs (AECC) as sole carbon source. Then, a novel adaptive laboratory evolution (ALE) approach was adapted to strengthen the n-butanol tolerance of *C. cellulovorans*, to fully utilize its n-butanol output potential. To further improve n-butanol production, both metabolic engineering and evolutionary engineering were combined, using the evolved strain as host for metabolic engineering. The n-butanol production from AECC of the engineered *C. cellulovorans* enhanced 138-fold from less than 0.025 g/L to 3.47 g/L. This method represents a milestone toward n-butanol production by CBP, using single recombinant clostridia. The engineered strain offers a promising CBP-enabling microbial chassis for n-butanol fermentation from lignocellulose.

Importance

Due to a lack of genetic tools, *Clostridium cellulovorans* DSM 743B has not been comprehensively explored as a putative strain platform for n-butanol production by consolidated bioprocessing (CBP). Based on the previous study of genetic tools, strain engineering of *C. cellulovorans* for the development of CBP-enabling microbial chassis was demonstrated in this study. Metabolic engineering and evolutionary

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55 engineering were integrated to improve the n-butanol production of *C. cellulovorans*
56 from the low-cost renewable agricultural waste of alkali-extracted, deshelled corn
57 cobs (AECC). The n-butanol production from AECC enhanced by 138-fold from less
58 than 0.025 g/L to 3.47 g/L, which represents the highest titer of n-butanol produced
59 using single recombinant clostridia by CBP reported to date. This engineered strain
60 serves as a promising n-butanol production chassis from lignocellulose by CBP.

61 **Keywords:** Clostridium, consolidated bioprocessing, metabolic engineering, adaptive
62 laboratory evolution, n-butanol

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64 **INTRODUCTION**

65 Due to the unsustainability of oil resources and global environmental
66 deterioration caused by the excessive use of fossil fuel, considerable attention has
67 recently been focused on lignocellulose refinery using a fermentation process for
68 various chemicals and fuels (1). A promising fermentation product for the substitution
69 of petroleum is butanol, which can be used both as an industrial commodity and a
70 gasoline substitute (2). Therefore, attention has focused on the production of butanol
71 from various lignocellulosic feedstocks by microbial fermentation. The traditional
72 process involves the synthesis of cellulases, saccharification, and hexose/pentose
73 co-utilization (3, 4). Alternatively, consolidated bioprocessing (CBP) can combine all
74 these processes within one step, which would decrease the cost of capital investment
75 for cellulase production, and thus offer the potential of an economical production of
76 butanol at industrial scale (5). However, no natural CBP-enabling microorganisms or
77 microbial consortia with the ability to produce butanol are currently available (6, 7).

78 The combination of both cellulolytic and butanol-producing phenotypes within a
79 single microorganism is an appealing challenge. A number of explorations have
80 attempted to engineer *E. coli* or yeast strains that directly produce
81 n-butanol/iso-butanol from lignocellulose. Achieved advances have been recently
82 reviewed (8-11). However, only a very low butanol titer could be obtained (8). An
83 alternative strategy is to develop native cellulolytic or solventogenic clostridia as
84 starting microbial chassis (12). Several key milestones have already been
85 accomplished in overexpressing cellulase encoding genes or the *in vivo* assembly of

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chimeric minicellulosome in solventogenic clostridia (13-15); however, these recombinant strains are still not capable to efficiently grow on lignocellulose. In contrast, introducing a butanol metabolic pathway into native cellulolytic clostridia represents a more meaningful progress toward the realization of CBP. The engineered *Clostridium thermocellum* has been reported to produce 5.4 g/L of iso-butanol from cellulose via a hybrid keto acid pathway (16). Similarly, other anaerobic cellulolytic bacteria such as *C. cellulolyticum* and *C. cellulovorans* (12, 17, 18), have also been suggested to have the potential to produce butanol by CBP after metabolic engineering.

C. cellulovorans DSM 743B is an anaerobic, celluloytic mesophile that can directly produce butyric acid as the main metabolic product from lignocellulose (19, 20). The complete sequencing and annotation of the genome deepened the understanding of both the plant cell wall degradation system and the metabolic network in *C. cellulovorans* (21). Studies on nuclease sequence analysis, cellulosome component properties, exoproteome profiles, and crystal structures of key cellulases have also achieved significant progress (22-25). Other breakthroughs on *C. cellulovorans* include genetic system development and genetic tool verification such as ClosTron and the CRISPR/Cas system, which provide a valuable foundation for metabolic engineering (6, 12, 26, 27). As a proof of concept, Yang et al. overexpressed an aldehyde/alcohol dehydrogenase (*adhE2*) in *C. cellulovorans*, which enabled the recombinant strain to produce 1.42 g/L n-butanol within 252 h (28). This represented the first metabolic engineering exemplified in *C. cellulovorans*; however, several

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other approaches remain unexplored, such as complicated genetic modification, n-butanol tolerance evolution, and process control optimization, which may promote the strain development of *C. cellulovorans*. In a previous study, Wen et al. developed a twin clostridial consortium, in which, *C. cellulovorans* DSM 743B was comprehensively engineered to promote the butyrate supply for *C. beijerinckii* NCIMB 8052 to achieve n-butanol production (6). However, the ability of *C. cellulovorans* to produce n-butanol was not exploited.

In this study, metabolic engineering and adaptive laboratory evolution were integrated to explore the n-butanol production of *C. cellulovorans* (pure culture) by CBP, as shown in Fig. 1. A CoA-dependent acetone-butanol-ethanol (ABE) pathway was introduced into *C. cellulovorans* to direct carbon flux from butyrate to n-butanol. Then, a novel evolutionary approach was developed to improve the n-butanol tolerance of *C. cellulovorans*, which may boost its n-butanol production potential. Finally, the ABE pathway was reconstructed in the evolved strains. The thus obtained strains produced far more butanol than unevolved recombinant strains.

MATERIALS AND METHODS

Strains, plasmids, primers, and strain cultivation

All bacterial strains, plasmids, and primers used in this study are listed in Tables 1, 2, and Fig. 2. *Escherichia coli* DH5α and ER2275 were aerobically cultivated at 37 °C in liquid or solidified Luria–Bertani (LB) media, which were composed of 5 g/L of yeast extract (AngelYeast Co., Ltd., China), 10 g/L of tryptone (AngelYeast Co.,

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Ltd., China), and 10 g/L of NaCl, and 1.5% agar (w/v) if necessary. Previously published protocols were employed for both the cultivation and genetic modification of *C. cellulovorans* DSM 743B (6). All stock culture was maintained in 25% glycerol and frozen at -80 °C.

DNA manipulation and vector construction

All DNA sequencing and oligonucleotide primers syntheses were conducted by Shanghai RuiDi Biological Technology Co., Ltd. (Shanghai, China). Previously described standard procedures were used to accomplish all recombinant DNA manipulations including genomic DNA preparation, DNA fragment amplification, digestion, ligation, transformation, colony PCR, plasmid extraction, and identification (6).

The construction of the ClosTron knock-out plasmid pWJ1-*spo0A* derived from pWJ1 and all overexpression (or complementation) plasmids derived from pXY1-P_{thl} were conducted according to previously published standard procedures (6).

Generation and screening of genetically modified strains

Electrocompetent cell preparation and electro-transformation of *C. cellulovorans* DSM 743B (from here on referred to as 743B) were conducted using previously described procedures (6). Positive transformants were screened and identified by colony PCR. Specially, for transformants that were generated from the electro-transformation of pWJ1-*spo0A*, PCR products of the desired mutants were approximately 0.9 kb longer than those of the wild type due to insertion of the group II intron (6). ClosTron knock-out plasmids were cured by repeated subculture on

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plates without erythromycin until a single colony that was sensitive to erythromycin had been isolated (6). Fig. 2 shows the derivative relationship of all genetically modified *C. cellulovorans* strains in this work.

Butanol tolerance evolutionary engineering and growth tolerance assay

Evolutionary engineering was accomplished by four stages with a stepwise increment of n-butanol (3, 6, 9, and 12 g/L) supplemented to the media. **ZQW10**: 743BΔs*po0A* and **ZQW00**: 743B were grown in tubes with 10 ml liquid medium in an anaerobic chamber (Thermo Forma, Inc., Waltham, MA, USA) at 37 °C. Cultures were inoculated to an OD_{600} of 0.3, grown for 24 h, and then harvested by centrifugation (3,000 × *g*, 2 min) for fresh inoculation. Evolution in media with higher n-butanol concentrations was not launched unless an OD_{600} of 1.0 was achieved within 24 h. Samples from the last batch were spread on solidified medium for clone selection.

Growth assay was used to evaluate the n-butanol tolerance of *C. cellulovorans*. The genetically modified or evolved *C. cellulovorans* were inoculated in media with various concentrations of n-butanol (0, 3, 6, 9, and 12 g/L), grown for 48 h. During this time, their OD_{600} were monitored and compared.

Batch fermentation of *C. cellulovorans* with constant pH and two-stage pH control

Alkali extracted corn cobs (AECC) were manufactured according to previously described protocol including alkali extraction, neutralization, thorough washing, and drying. The particles varied from 30-40 mesh (0.45-0.60 mm). Cellulose,

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hemicellulose, and lignin contents were 69.8, 27.4, and 1.47% (w/w), respectively (6). All batch fermentations with glucose or AECC as the sole carbon source for phenotype comparison (expressed as bar charts) were conducted in 500 mL anaerobic shaken flasks (customized from Guxin biotech Co., Ltd., Shanghai, China) with a 400-mL working volume. All batch fermentations for the study of the metabolic progress (expressed as line chart) were conducted in a 3 L BioFlo 110 bioreactor (New Brunswick Scientific Co., Inc., New Jersey, USA) with a 1.6 L working volume. The pH was controlled via automatic addition of 5 N NaOH, while temperature and stirring speed were kept at 37 °C and 150 rpm, respectively. The media were supplemented with 15 mg/L erythromycin for mutants that harbor pXY1 or pWJ1 series plasmids. Samples were collected in an anaerobic chamber at regular intervals and enzyme activity, biomass, as well as substrate and product concentration were analyzed. The inoculum size of *C. cellulovorans* DSM 743B was 10% (v/v) if not indicated otherwise (29).

Analysis

A DU730 spectrophotometer (Beckman Colter) was used to monitor cell growth on glucose with an optical density of 600 nm (OD_{600}). The cell mass on AECC particles and the AECC concentration were calculated following previously reported procedures (29). Volatile solvents and organic acids (ethanol, n-butanol, acetone acetate, and butyrate) were determined by gas chromatography (7890A, Agilent, Wilmington, DE, USA), while lactate, monosaccharides, and disaccharides were measured by a high-performance liquid chromatography (HPLC) system (1200 series;

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196 Agilent, Wilmington, DE, USA) (6). The assay of butyraldehyde dehydrogenase and
197 butanol dehydrogenase activities in **ZQW01**, **ZQW02**, and **ZQW03** were
198 accomplished following the protocol of Yang et al. except that the crude cell extract
199 was obtained using a French press (Constant Systems Limited, UK) (28). The carbon
200 recovery of C_2 + C_3 + C_4 products (acetate, ethanol, lactate, butyrate, n-butanol, and
201 acetone) was calculated as previously described (6).
202
203 **RESULTS**
204 **Heterologous *adhE1* introduction enables *C. cellulovorans* to produce n-butanol**
205 According to the prediction of KEGG, a complete CoA-dependent butanol
206 synthesis pathway exists in *C. cellulovorans*. However, only less than 0.025 g/L of
207 n-butanol can be detected in broth (30). Low endogenous alcohol/aldehyde
208 dehydrogenase activity was confirmed to be a bottleneck for n-butanol production
209 (28). It has been reported that *adhE1* overexpression (encoding an alcohol/aldehyde
210 dehydrogenase in *C. acetobutylicum*) can restore the n-butanol production to wild
211 type levels in the *C. acetobutylicum* M5 strain (a non-sporulating,
212 non-solvent-producing mutant without the pSOL1 megaplasmid containing
213 alcohol/aldehyde dehydrogenase and the acetone-formation genes) (31, 32).
214 Accordingly, this study attempted to refactor the n-butanol pathway in *C.*
215 *cellulovorans* by introducing alcohol/aldehyde dehydrogenase genes.
216 There are two homologous *adhE* genes: *adhE1* (*CA_p0162*, located in the *sol*
217 operon *orfL-adhE1-ctfA-ctfB*) and *adhE2* (*CA_p0035*, a monocistronic operon) in the

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C. acetobutylicum ATCC 824 (33, 34). The effect of overexpressing either of these two genes on the n-butanol production was evaluated (Fig. 3A). **ZQW02**: 743B(pXY1-P_{thl}-*adhE2*) produced 0.37 g/L n-butanol with AECC as the sole carbon source within 108 h. However, the titer of n-butanol produced by **ZQW03**: 743B(pXY1-P_{thl}-*adhE1*) reached 1.63 g/L, which is almost four-fold of that achieved by **ZQW02**. The butyraldehyde dehydrogenase activity and the n-butanol dehydrogenase activity in **ZQW03** were 88.2% and 152% higher than in **ZQW02**, and 7.97 and 13.6-fold higher than in **ZQW01**: 743B(pXY1-P_{thl}) (as control), which indicates why the n-butanol output by **ZQW03** was higher than by **ZQW02** (Fig. 3B).

Then, the fermentation progress of **ZQW03** was explored in a 3 L bioreactor with **ZQW01** as control (Fig. 3C, 3D, and Fig. S1). Less butyrate accumulated in the broth by **ZQW03** compared to **ZQW01**. Moreover, n-butanol synthesis by **ZQW03** coincides with butyrate accumulation and butyrate hardly decreased in the fermentation anaphase, indicating that butyrate was almost not re-assimilated by **ZQW03** itself. Within 108 h, **ZQW03** produced 1.64 g/L n-butanol, 0.635 g/L ethanol from 37.2 g/L AECC with 5.52 g/L butyrate, and 8.78 g/L total sugars (mainly pentose) residual in the broth. These results represent an acetone-uncoupled n-butanol production process by CBP, which indicates that *C. cellulovorans* has potential in producing biofuel mixtures with high n-butanol ratio as previously suggested (35, 36).

Butyrate re-assimilation by overexpressing *ctfAB-adc* to drive n-butanol production

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According to the working hypothesis, n-butanol titer and yield may be further promoted by re-assimilating and transferring the butyrate accumulated by **ZQW02** and **ZQW03** to n-butanol. In *C. acetobutylicum*, it has been reported that the bifunctional BK-I encoded by *bukI* (*CA_c3075*) not only catalyzes butyrate formation, but is also responsible for the uptake of butyrate without simultaneous production of acetone (37, 38). Therefore, *bukI* from *C. acetobutylicum 824* was overexpressed coupled with *adhE1* in *C. cellulovorans*. However, the n-butanol output of **ZQW07**: 743B(pXY1-P_{thl}-*buk*-P_{thl}-*adhE1*) did not noticeably change, while the residual butyrate increased by 32.6% (Fig. 4A). This indicates a much higher efficiency of butyrate synthesis than the re-assimilation in *C. cellulovorans*. This further suggests that the knockout of endogenous *buk* in *C. cellulovorans* may help to direct the butyrate flux to n-butanol formation; however, a *buk* (*Clocel_3674*, encoding butyrate kinase) deficient mutant of *C. cellulovorans* could not be generated using ClosTron even with long-term screening.

Alternatively, a routine strategy for butyrate re-assimilation in *C. acetobutylicum* and *C. beijerinckii* was used under solventogenesis. The strategy of overexpressing genes involved in CoA transferase encoded by *ctfA-ctfB* (*CA_p0163-0164* or *Cbei_3833-3834*) has been successfully verified in *C. acetobutylicum*, *C. tyrobutyricum*, and *C. beijerinckii* (6, 39, 40). Both the *adhE1* and *ctfA-ctfB* genes located in *C. acetobutylicum*'s sol operon were introduced, followed by its adjacent *adc* gene (*CA_p0165*, with native promoter) (34), since there are no known genes involved in the acetoacetate metabolism of *C. cellulovorans*. Interestingly, the

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heterologous ABE (acetone-butanol-ethanol) pathway worked very well in **ZQW05**: 743B(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*). Compared to **ZQW03**, the n-butanol output of **ZQW05** was enhanced by 49.3%, while residual butyrate and acetate decreased by 13.2% and 17.3%, respectively (Fig. 4A). It is worth noting that the overexpression of *ctfAB-adc* (without *adhE1*) in wild-type *C. cellulovorans* showed no improvement of n-butanol production. This indirectly supports the hypothesis that low alcohol/aldehyde dehydrogenase activity maybe a bottleneck for the n-butanol synthesis in wild-type *C. cellulovorans.*

Furthermore, the fermentation progress of **ZQW05** was investigated in a 3 L bioreactor (Fig. 4B, 4C, and 4D). Within 108 h, **ZQW05** produced 2.27 g/L n-butanol and 0.712 g/L ethanol from 38.7 g/L AECC. This was not a typical two-phase fermentation of acidogenesis followed by solventogenesis as in solventogenic clostridia (41). n-Butanol production coincides with butyrate accumulation during the first 72 h, after which, acetone was also produced. Simultaneously, acetate and butyrate decreased by 8% and 5.1% from their peak titer, which were inferred to be re-assimilated by the engineered *C. cellulovorans* harboring *ctfA-ctfB* gene (Fig. 4B). During the fermentation anaphase, AECC degradation, total sugar utilization, and n-butanol production almost ceased. This was in agreement with the results of Yang et al. when their engineered *C. cellulovorans* was cultured with glucose or cellobiose as the sole carbon source (28).

Adaptive laboratory evolution to improve the n-butanol tolerance of *C.*

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284 ***cellulovorans***

285 Butanol toxicity is a main barrier for solvent production in Clostridium (mainly
286 butanol) with high titer since it may cause microbial growth inhibition and cell death
287 (42). Improvement in butanol tolerance sometimes leads to an increase in butanol
288 production (43). The cellular responses and molecular mechanisms that can overcome
289 butanol stress in Clostridium are complex and not yet fully understood (44).
290 Laboratory adaptive evolution has been shown to be an efficient strategy for the
291 improvement of butanol tolerance (45, 46). In this study, two evolutionary approaches
292 were adapted to boost the n-butanol tolerance of *C. cellulovorans* to improve
293 n-butanol production.

294 Wild-type **ZQW00**: 743B and recombinant **ZQW10**: 743BΔ*spo0A* were chosen
295 as parent strains for parallel adaptive evolution experiments. Both were cultured in
296 media supplemented with a serial enrichment of n-butanol (from 3 g/L to 12 g/L) to
297 promote evolution. As expected, the culture concomitantly adapted to these intensified
298 conditions. It took 17, 15, and 22 days for **ZQW10** to evolve so that it could
299 successfully resist 3 g/L, 6 g/L, and 9 g/L of butanol, respectively (Fig. 5A); however,
300 for **ZQW00**, it took 11, 11, and 25 days, respectively (Fig. S2). The offspring of
301 **ZQW10** needed about 29 days to achieve a stable biomass above 1.0 (OD_{600}), while
302 **ZQW00** could not further evolve in media with added 12 g/L of n-butanol (Fig. 5A
303 and Fig. S2).

304 After four rounds of evolution over a total of 83 days, the resulting evolved
305 **ZQW10** and **ZQW00** were harvested and spread on plates that were supplemented

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with 12 g/L and 9 g/L n-butanol (because the evolved **ZQW00** cannot grow well in media adding 12 g/L of n-butanol), respectively. The phenotype of several evolved colonies and selected strains **ZQW30**: 743BΔs*po0A**11 (derived from **ZQW10**) and **ZQW20**: 743B*4 (derived from **ZQW00**) were tested for further assessment and engineering (Fig. S3). With an initial 12 g/L of n-butanol in the medium, the 48-h biomass of **ZQW30** achieved 45.2% of that cultured in blank Clocel medium (without n-butanol), while **ZQW10** did not show noticeable growth. The n-butanol tolerance of mutants derived from **ZQW00**, **ZQW10**, **ZQW20**, and **ZQW30** was evaluated (Fig. 5B and Fig. S4). In general, the strains derived from **ZQW30** can resist more butanol than those derived from **ZQW20**. Since **ZQW35** can resist more butanol than **ZQW25**, **ZQW35** was selected for the subsequent exploration (Fig. 5B).

Spo0A inactivation seems to contribute to strain evolution but weakens the AECC degradation by **ZQW10** and **ZQW30** (Fig. S5). This may be because the *spo0A* deficient mutants were prone to cell lysis (47, 48). The *spo0A* gene was complemented using a plasmid-based expression, driven by a *thl* promoter (49) in **ZQW10** and **ZQW30**. Both **ZQW11**: 743BΔs*po0A*(pXY1-P_{thl}-s*po0A*) and **ZQW31**: 743BΔs*po0A**11(pXY1-P_{thl}-s*po0A*) restored their AECC degradation ability to a level similar to that of the wild-type (Fig. S5). Accordingly, *spo0A* complementation was implemented in all mutants derived from **ZQW10** and **ZQW30** and *spo0A* complementation did not strongly affect n-butanol tolerance (Fig. S4).

Combination of metabolic and evolutionary engineering to improve n-butanol production

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We decided to include more advantageous genetic modifications as described above in the evolved strains toward a further improvement of n-butanol production. The metabolic profiles were compared among mutants derived from different parent strains (Figs. 2 and 6A). The n-butanol output of **ZQW33**: 743BΔs*spo0A**11(pXY1-P_{thl}-*adhE1*-P_{thl}-s*po0A*) and **ZQW35**: 743BΔs*spo0A**11(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-s*po0A*) increased by 52.2% and 50.5% compared to **ZQW13**: 743BΔs*spo0A*(pXY1-P_{thl}-*adhE1*-P_{thl}-s*po0A*) and **ZQW15**: 743BΔs*spo0A*(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-s*po0A*), respectively. This indicates that the reinforced n-butanol tolerance improved n-butanol production.

Batch fermentation in a 3 L bioreactor was performed to investigate the metabolic profile of **ZQW35**. During the first 36 h, no obvious phenotype difference was found between strains before and after evolution. However, AECC degradation, butyrate re-assimilation, and n-butanol production improved during the fermentation anaphase, suggesting relatively higher cell viability of **ZQW35** than **ZQW05**. During 96 h, **ZQW35** produced 3.02 g/L n-butanol and 0.777 g/L ethanol from 40.2 g/L AECC with 4.19 g/L of residual butyrate (Fig. 6B, 6C, and 6D). Compared to **ZQW05**, the n-butanol production increased by 33% and the residual butyrate decreased by 19.8%.

A two-stage pH control strategy was proposed to coordinate native cellular growth (pH of approximately 7.0) (28, 30) and cellulase production with the heterologous pathway of n-butanol synthesis and butyrate re-assimilation (pH 4.5-6.0) in *C. cellulovorans* (29) (34, 41). This further improved the n-butanol production. In such a strategy, pH was controlled at 7.0 during the first 48 h, and then decreased to

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6.0. In anaerobic shaken flasks, the n-butanol production of **ZQW13**, **ZQW33**, **ZQW15**, and **ZQW35** increased by 22.4%, 17.3%, 19.1%, and 13.3%, respectively. Simultaneously, their butyrate secretion decreased by 16.3%, 5%, 23.4%, and 15.6%, respectively (Fig. 7A).

A two-stage pH control strategy was implemented in a 3 L bioreactor to maximize the butanol output of **ZQW35** (Fig. 7B, 7C, and 7D). Compared to the fermentation behavior shown in Fig. 6B, both the n-butanol accumulation and butyrate re-assimilation clearly accelerated between 48 and 72 h, but almost ceased after 84 h. The batch fermentation can be stopped 12 h earlier than that in Fig. 6B. During 84 h, **ZQW35** produced 3.47 g/L n-butanol and 0.875 g/L ethanol from 43.1 g/L AECC with 3.76 g/L butyrate and 9.53 g/L residual total sugars (mainly xylose and arabinose) in the broth (Fig. 7B, 7C, and 7D). The n-butanol production increased by 14.9% and the n-butanol productivity increased by 31.3% compared to that obtained under a control of constant neutral pH. This supports a beneficial two-stage pH control strategy. The n-butanol titer and productivity here were 2.44-fold and 7.32-fold higher than those obtained in a previous study (28).

Discussion

In the present study, metabolic engineering and adaptive laboratory evolution were integrated to improve the n-butanol production of *C. cellulovorans* DSM 743B from AECC, a low-cost and renewable agricultural waste. This represents an application of a previous study of genetic tools of *C. cellulovorans* DSM 743B in

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372 CBP-enabling microbial chassis development (6).

373 To enable *C. cellulovorans* DSM 743B to produce n-butanol, the CoA-dependent
374 ABE pathway, including *adhE1* and *ctfAB-adc* genes from *C. acetobutylicum* ATCC
375 824, were introduced. The metabolically engineered strains showed increased butanol
376 output compared to that of Yang *et al.*, who only overexpressed *adhE2 in C.*
377 *cellulovorans*, and eventually achieved 1.42 g/L n-butanol production from cellulose
378 (28). It is interesting that **ZQW03**: 743B(pXY1-P_{thl}-*adhE1*) exhibited higher
379 n-butanol output, butyraldehyde dehydrogenase activity, and butanol dehydrogenase
380 activity compared to **ZQW02:** 743B(pXY1-P_{thl}-*adhE2*) (Fig. 3B). The higher butanol
381 dehydrogenase activity in **ZQW03** may be attributed to *C. cellulovorans* itself. Since
382 *C. cellulovorans* possesses several putative butanol dehydrogenase encoding genes
383 (*Clocel_1140*, *Clocel_1949*, *Clocel_4197*, *Clocel_2402*, and *Clocel_3817*), it is likely
384 that one of them might be induced by the butyraldehyde generated by the
385 heterogenous AdhE1. A similar phenomenon has been demonstrated in *C.*
386 *acetobutylicum* under solventogenesis, with the exception that the butanol
387 dehydrogenases expressed in *C. acetobutylicum* are NADPH dependent (33).

388 Previous studies confirmed Spo0A as a transcriptional regulator that is closely
389 related to sporulation, solvent production, and butanol tolerance in Clostridium (50,
390 51). It plays a central role by mainly regulating sporulation-specific sigma factors,
391 solvent genes, and heat shock protein genes (52). Overexpression of *spo0A* can
392 enhance the solvent tolerance of Clostridium, thus increasing its solvent yield;
393 however, this leads to the early onset of sporulation (50, 51).

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394 We hypothesized that there may be further molecular mechanisms that lead to
395 stress-resistance in *C. cellulovorans*, such as several two-component systems or
396 membrane transport protein, that are not entirely dependent on *spo0A* (53, 54). When
397 *spo0A* is knocked out, attenuated, or mutated, other mechanisms may be activated or
398 reinforced to strain the response to butanol stress. Accordingly, two parallel
399 evolutionary approaches that use *spo0A*-knockout strain **ZQW10** and wild-type
400 **ZQW00** as original strains, respectively, were investigated and compared.

401 The evolved *spo0A*-deficient strain **ZQW30** was demonstrated to tolerate 12 g/L
402 of butanol after four rounds or a total of 83 days of domestication. This implied
403 putative molecular machines independent of *spo0A* in *C. cellulovorans* that resist such
404 high butanol concentration. Comparative analysis of genome re-sequencing,
405 transcriptional analysis, and functional genomics studies of **ZQW30**, **ZQW20**, and
406 **ZQW00**, coupled with subsequent genetic and biochemical validation experiments,
407 are currently being conducted to investigate the underlying molecular mechanism that
408 is responsible for the higher n-butanol resistance in **ZQW20** and **ZQW30**. The
409 evolutionary approach based on *spo0A*-knockout strain offers a new perspective for
410 the exploration of diversities of stress response mechanism in Clostridium.

411 In addition, *spo0A* inactivation helps to filter false positives benefiting from
412 *spo0A* related stress mechanisms, which leads to a more efficient and robust adaptive
413 laboratory evolution. The inactivation of *spo0A* weakens the stress-adaptability of
414 Clostridium, which means that Clostridium is unable to survive (or resist butanol) by
415 forming spores or by regulating *spo0A* expression levels (thus regulating target genes)

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(52). These circumstances drive the strain to either evolve or die. As shown in Fig. S3, far more survivors derived from **ZQW10** than those derived from **ZQW00** have real butanol tolerance phenotype. Therefore, **ZQW10** evolved more smoothly than **ZQW00**, and the n-butanol tolerance of the strains derived from **ZQW30** (evolved **ZQW10**) was generally superior to those derived from **ZQW20** (evolved **ZQW00**) (see Fig. 5B and Fig. S4).

In this respect, the inactivation of genes involved in sporulation such as *spo0A* in *C. cellulovorans* provides a novel and reliable approach for adaptive laboratory evolution toward enhanced butanol tolerance of Clostridium, which may be extended to the study of lignocellulosic hydrolysate resistance.

The reinforced n-butanol tolerance indeed improved the n-butanol production from 2.27 g/L of **ZQW05** to 3.02 g/L of **ZQW35** (Figs. 4B and 6B). However, the toxicity of 3 g/L n-butanol no longer significantly affected the fermentation phenotype of mutants derived from **ZQW20** and **ZQW30** (Fig. 5B). This provides an indication why butanol output did not differ strongly between **ZQW35** and **ZQW25**: 743B*4(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*), or between **ZQW33** and **ZQW23**: 743B*4(pXY1-P_{thl}-*adhE1*) (Fig. 6A and Fig. S6). These results imply that n-butanol inhibition can limit the n-butanol output of the unevolved *C. cellulovorans*; however, this seems to not be the essential determinant for the achievement of high n-butanol titer.

The integration of metabolic and evolutionary engineering offered a stepwise promotion of n-butanol output (Table S1 and Fig. S7), which accelerated the overall

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development of a *C. cellulovorans* CBP-enabling microbial chassis for increased n-butanol production using lignocellulose or glucose as the sole carbon source. The n-butanol production of *C. cellulovorans* using AECC could be enhanced by 138-fold from less than 0.025 g/L to 3.47 g/L (Fig. 8). Among CBPs for n-butanol production with single recombinant Clostridia using various lignocellulosic biomasses, the presented results represent a significant progress in n-butanol titers and productivity (Tab. 3). In addition, the evolved strains may offer indications for new n-butanol tolerance mechanisms or target genes for reverse metabolic engineering. The engineered *C. cellulovorans* offers a promising microbial chassis for the n-butanol production from lignocellulose by CBP.

However, the n-butanol titer and ratio in this study still offer room for improvement. As shown in Figs. 7B and 7D, 3.76 g/L of butyrate and 6.16 g/L of xylose residual remained in the broth at the end of fermentation (84 h), which should ideally have been converted to butanol. To either avoid or decrease butyrate production, endogenous *buk* knockout was attempted in *C. cellulovorans* using ClosTron. However, no mutant of *C. cellulovorans* with inactivated *buk* was obtained. This may be due to an inherent limitation of group II intron technology, the inactivation efficiency of which is gene specific (or dependent) (55, 56). In other studies, the *buk* gene in *C. acetobutylicum* ATCC *824* has been successfully inactivated using in-frame deletion methods; consequently, *buk* inactivation directed carbon flux from butyrate to butanol (57, 58). A similar approach may work for *C. cellulovorans*. With regard to xylose, metabolic engineering is ideally suited for *C.*

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460 *cellulovorans* to alleviate carbon catabolite repression (CCR) and promote butanol
461 conversion. The reason for this is that insufficient uptake and utilization of xylose
462 leads to slow growth and excessively long fermentation. These strategies such as
463 overexpression of xylose symporter (XylT) and inactivation of xylose operon
464 transcriptional repressor (XylR), have been proved successful in *C. beijerinckii* and *C.*
465 *tyrobutyricum* (49, 59).

466 Similar than in the achievements reported for other Clostridia, ABE pathway
467 engineering (33, 58), pentose metabolic engineering (49, 59), cellulase engineering
468 (13), and understanding the resistance mechanism of inhibitors (42, 43, 53) will likely
469 promote the further development of the *C. cellulovorans* platform more effectively.

470

471

472 **REFERENCES**

473 1. Ren C, Wen Z, Xu Y, Jiang W, Gu Y. 2016. Clostridia: a flexible microbial platform for the
474 production of alcohols. Curr Opin Chem Biol 35:65-72.
475 2. Chen CT, Liao JC. 2016. Frontiers in microbial 1-butanol and isobutanol production. FEMS
476 Microbiol Lett 363.
477 3. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C,
478 Panning B, Ploegh HL, Bassik MC, Qi LS, Kampmann M, Weissman JS. 2014. Genome-scale
479 CRISPR-mediated control of gene repression and activation. Cell 159:647-61.
480 4. Jiang Y, Liu J, Jiang W, Yang Y, Yang S. 2015. Current status and prospects of industrial
481 bio-production of n-butanol in China. Biotechnol Adv 33:1493-1501.
482 5. Olson DG, McBride JE, Shaw AJ, Lynd LR. 2012. Recent progress in consolidated
483 bioprocessing. Curr Opin Biotechnol 23:396-405.
484 6. Wen Z, Minton NP, Zhang Y, Li Q, Liu J, Jiang Y, Yang S. 2017. Enhanced solvent production
485 by metabolic engineering of a twin-clostridial consortium. Metab Eng 39:38-48.
486 7. Brethauer S, Studer MH. 2014. Consolidated bioprocessing of lignocellulose by a microbial
487 consortium. Energ Environ Sci 7:1446.
488 8. Salehi Jouzani G, Taherzadeh MJ. 2015. Advances in consolidated bioprocessing systems for
489 bioethanol and butanol production from biomass: a comprehensive review. Biofuel Research J
490 2:152-195.
491 9. Hasunuma T, Ishii J, Kondo A. 2015. Rational design and evolutional fine tuning of

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Applied and Environmental Microbiology
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Saccharomyces cerevisiae for biomass breakdown. Curr Opin Chem Biol 29:1-9.

10. den Haan R, van Rensburg E, Rose SH, Goergens JF, van Zyl WH. 2015. Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. Curr Opin Biotechnol 33:32-38.
11. Yamada R, Hasunuma T, Kondo A. 2013. Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnol Adv 31:754-763.
12. Wang J, Yang X, Chen C-C, Yang S-T. 2014. Engineering clostridia for butanol production from biorenewable resources: from cells to process integration. Curr Opin Chem Biol 6:43-54.
13. Willson BJ, Kovacs K, Wilding-Steele T, Markus R, Winzer K, Minton NP. 2016. Production of a functional cell wall-anchored minicellulosome by recombinant *Clostridium acetobutylicum* ATCC 824. Biotechnol Biofuels 9:109.
14. Kovács K, Willson BJ, Schwarz K, Heap JT, Jackson A, Bolam DN, Winzer K, Minton NP. 2013. Secretion and assembly of functional mini-cellulosomes from synthetic chromosomal operons in *Clostridium acetobutylicum* ATCC 824. Biotechnol Biofuels 6:117.
15. Mingardon F, Chanal A, Tardif C, Fierobe HP. 2011. The issue of secretion in heterologous expression of *Clostridium cellulolyticum* cellulase-encoding genes in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 77:2831-2838.
16. Lin PP, Mi L, Morioka AH, Yoshino KM, Konishi S, Xu SC, Papanek BA, Riley LA, Guss AM, Liao JC. 2015. Consolidated bioprocessing of cellulose to isobutanol using *Clostridium thermocellum*. Metab Eng 31:44-52.
17. Gaida SM, Liedtke A, Jentges AH, Engels B, Jennewein S. 2016. Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose. Microb Cell Fact 15:6.
18. Higashide W, Li Y, Yang Y, Liao JC. 2011. Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. Appl Environ Microbiol 77:2727-33.
19. Shoseyov O, Doi RH. 1990. Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. Proc Natl Acad Sci U S A 87:2192-5.
20. Shoseyov O, Takagi M, Goldstein MA, Doi RH. 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. Proc Natl Acad Sci U S A 89:3483-7.
21. Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Kawade Y, Yamamoto K, Uemura M, Fujita Y, Doi RH, Ueda M. 2010. Genome sequence of the cellulosome-producing mesophilic organism *Clostridium cellulovorans* 743B. J Bacteriol 192:901-2.
22. Xu CG, Huang RR, Teng L, Jing XY, Hu JQ, Cui GZ, Wang YL, Cui Q, Xu J. 2015. Cellulosome stoichiometry in *Clostridium cellulolyticum* is regulated by selective RNA processing and stabilization. Nat Commun 6.
23. Jeon SD, Kim SJ, Park SH, Choi G-W, Han SO. 2015. Hydrolytic effects of scaffolding proteins CbpB and CbpC on crystalline cellulose mediated by the major cellulolytic complex from *Clostridium cellulovorans*. Bioresour Technol 191:505-11.
24. Bianchetti CM, Brumm P, Smith RW, Dyer K, Hura GL, Rutkoski TJ, Phillips GN. 2013. Structure, dynamics, and specificity of endoglucanase D from *Clostridium cellulovorans*. J Mol Biol 425:4267-4285.
25. Morisaka H, Matsui K, Tatsukami Y, Kuroda K, Miyake H, Tamaru Y, Ueda M. 2012. Profile of native cellulosomal proteins of *Clostridium cellulovorans* adapted to various carbon

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sources. AMB express 2:1-5.

26. Zhou YJ, Buijs NA, Zhu Z, Gomez DO, Boonsombuti A, Siewers V, Nielsen J. 2016. Harnessing yeast peroxisomes for biosynthesis of fatty-acid-derived biofuels and chemicals with relieved side-pathway competition. J Am Chem Soc 138:15368-15377.

27. Yang X, Xu M, Yang ST. 2016. Restriction modification system analysis and development of in vivo methylation for the transformation of *Clostridium cellulovorans*. Appl Microbiol Biotechnol 100:2289-99.

28. Yang X, Xu M, Yang ST. 2015. Metabolic and process engineering of *Clostridium cellulovorans* for biofuel production from cellulose. Metab Eng 32:39-48.

29. Wen Z, Wu M, Lin Y, Yang L, Lin J, 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.

30. Sleat R, Mah RA, Robinson R. 1984. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl Environ Microbiol 48:88-93.

31. Lee JY, Jang Y-S, Lee J, Papoutsakis ET, Lee SY. 2009. Metabolic engineering of *Clostridium acetobutylicum* M5 for highly selective butanol production. Biotechnol J 4:1432-40.

32. Sillers R, Chow A, Tracy B, Papoutsakis ET. 2008. Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab Eng 10:321-332.

33. Yoo M, Croux C, Meynial-Salles I, 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.

34. Durre P. 2008. Fermentative butanol production: bulk chemical and biofuel. Ann N Y Acad Sci 1125:353-62.

35. Gong F, Bao G, Zhao C, Zhang Y, Li Y, Dong H. 2016. Fermentation and genomic analysis of acetone-uncoupled butanol production by *Clostridium tetanomorphum*. Appl Microbiol Biotechnol 100:1523-9.

36. Sabra W, Groeger C, Sharma PN, Zeng AP. 2014. Improved n-butanol production by a non-acetone producing *Clostridium pasteurianum* DSMZ 525 in mixed substrate fermentation. Appl Microbiol Biotechnol 98:4267-76.

37. Jang YS, Im JA, Choi SY, Lee JI, Lee SY. 2014. Metabolic engineering of *Clostridium acetobutylicum* for butyric acid production with high butyric acid selectivity. Metab Eng 23:165-74.

38. Lehmann D, Radomski N, Luetke-Eversloh T. 2012. New insights into the butyric acid metabolism of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 96:1325-1339.

39. Yu L, Zhao J, Xu M, Dong J, Varghese S, Yu M, Tang IC, Yang S-T. 2015. Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production: effects of CoA transferase. Appl Microbiol Biotechnol 99:4917-4930.

40. Jang YS, Lee JY, Lee JM, Lee SY. 2009. Metabolic engineering of *Clostridium acetobutylicum* M5 for butanol-ethanol production by complementation of *adhE1* and *ctfAB* genes. New Biotechnol 25:S273-S274.

41. Moon HG, Jang Y-S, Cho C, Lee J, Binkley R, Lee SY. 2016. One hundred years of clostridial

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butanol fermentation. FEMS Microbiol Lett 363.

42. Peabody GL, Kao KC. 2016. Recent progress in biobutanol tolerance in microbial systems with an emphasis on Clostridium. FEMS Microbiol Lett 363:fnw017.

43. Nicolaou SA, Gaida SM, Papoutsakis ET. 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 12:307-331.

44. Liu S, Qureshi N, Hughes SR. 2017. Progress and perspectives on improving butanol tolerance. World Journal of Microbiology & Biotechnology 33.

45. Dragosits M, Mattanovich D. 2013. Adaptive laboratory evolution - principles and applications for biotechnology. Microb Cell Fact 12.

46. Yang S-T, Zhao J. 2013. Adaptive engineering of *Clostridium* for increased butanol production. US Patent 8450093.

47. Li Y, Xu T, Tschaplinski TJ, Engle NL, Yang Y, Graham DE, He Z, Zhou J. 2014. Improvement of cellulose catabolism in *Clostridium cellulolyticum* by sporulation abolishment and carbon alleviation. Biotechnol Biofuels 7.

48. Kodama T, Endo K, Ara K, Ozaki K, Kakeshita H, Yamane K, Sekiguchi J. 2007. Effect of *Bacillus subtilis* spo0A mutation on cell wall lytic enzymes and extracellular proteases, and prevention of cell lysis. J Biosci Bioeng 103:13-21.

49. Xiao H, Li Z, Jiang Y, Yang Y, Jiang W, Gu Y, Yang S. 2012. Metabolic engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. Metab Eng 14:569-78.

50. Harris LM, Welker NE, Papoutsakis ET. 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. J Bacteriol 184:3586-3597.

51. Alsaker KV, Spitzer TR, Papoutsakis ET. 2004. Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. J Bacteriol 186:1959-1971.

52. Al-Hinai MA, Jones SW, Papoutsakis ET. 2015. The Clostridium sporulation programs: diversity and preservation of endospore differentiation. Microbiol Mol Biol R 79:19-37.

53. Xu M, Zhao J, Yu L, Tang IC, Xue C, Yang S-T. 2015. Engineering *Clostridium acetobutylicum* with a histidine kinase knockout for enhanced n-butanol tolerance and production. Appl Microbiol Biotechnol 99:1011-1022.

54. Liu J, Lin Q, Chai X, Luo Y, Guo T. 2018. Enhanced phenolic compounds tolerance response of *Clostridium beijerinckii* NCIMB 8052 by inactivation of *Cbei_3304*. Microb Cell Fact 17.

55. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. 2007. The ClosTron: A universal gene knock-out system for the genus Clostridium. J Microbiol Methods 70:452-464.

56. Enyeart PJ, Mohr G, Ellington AD, Lambowitz AM. 2014. Biotechnological applications of mobile group II introns and their reverse transcriptases: gene targeting, RNA-seq, and non-coding RNA analysis. Mobile DNA 5.

57. Green EM, Boynton ZL, Harris LM, Rudolph FB, Papoutsakis ET, Bennett GN. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiol (Reading, England) 142 (Pt 8):2079-86.

58. Yoo M, Croux C, Meynial-Salles I, Soucaille P. 2017. Metabolic flexibility of a butyrate pathway mutant of *Clostridium acetobutylicum*. Metab Eng 40:138-147.

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59. Fu H, Yang S-T, Wang M, Wang J, Tang IC. 2017. Butyric acid production from lignocellulosic biomass hydrolysates by engineered *Clostridium tyrobutyricum* overexpressing xylose catabolism genes for glucose and xylose co-utilization. Bioresour Technol 234:389-396.

60. Mermelstein L, Papoutsakis E. 1993. In vivo methylation in Escherichia coli by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 59:1077-1081.

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

62. Xiao H, Gu Y, Ning Y, Yang Y, Mitchell WJ, Jiang W, 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 77:7886-95.

63. Chanal A, Mingardon F, Bauzan M, Tardif C, Fierobe HP. 2011. Scaffoldin modules serving as "cargo" domains to promote the secretion of heterologous cellulosomal cellulases by *Clostridium acetobutylicum*. Appl Environ Microbiol 77:6277-80.

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TABLE 1 Strains and plasmids used in this study.

Strains or plasmids	Description of genotype	Source or reference
Strains		
E. coli DH5α	Commercial transformation host	GIBCO BRL, Life Technologies
E. coli ER2275	Strain used to pre-methylate plasmids for electron-transformation in *C. acetobutylicum* ATCC 824 and *C. cellulovorans* DSM 743B	New England Biolabs
C. acetobutylicum ATCC 824	Wild type	ATCC
ZQW00: *C. cellulovorans* DSM 743B	Wild type	DSMZ
ZQW01: 743B(pXY1-P_{thl})	743B/pXY1- P_{thl}	(6)
ZQW02: 743B(pXY1-P_{thl}-*adhE2*)	743B/pXY1-P_{thl}-*adhE1*	This study
ZQW03: 743B(pXY1-P_{thl}-*adhE1*)	743B/ pXY1-P_{thl}-*adhE2*	This study
ZQW04: 743B(pXY1-P_{thl}-*ctfAB-adc*)	743B/pXY1-P_{thl}-*ctfAB-adc*	This study
ZQW05: 743B(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*)	743B/pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*	This study
ZQW06: 743B(*pXY1-*P_{thl}-*bukI*)	743B/pXY1-P_{thl}-*bukI*	This study
ZQW07: 743B(pXY1-P_{thl}-*bukI*-P_{thl}-*adhE1*)	743B/pXY1-P_{thl}-*bukI*-P_{thl}-*adhE1*	This study
ZQW11: 743B(pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*)	743B/pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*	This study
ZQW10: 743BΔ*spo0A*	743B *spo0A*::intron	(6)
ZQW11: 743BΔ*spo0A*(pXY1-P_{thl}-*spo0A*)	743B *spo0A*::intron/pXY1-P_{thl}-spo0A	(6)
ZQW13: 743BΔ*spo0A*(pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*)	743B *spo0A*::intron/pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*	This study
ZQW15:743BΔ*spo0A*(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*)	743B *spo0A*::intron/pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*	This study
ZQW20: 743B*4	Evolved strain from wild **ZQW00**	This study
ZQW23:743B*4(pXY1-P_{thl}-*adhE1*)	743B*4/pXY1-P_{thl}-*adhE1*	This study
ZQW25: 743B*4(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*)	743B*4/pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*	This study
ZQW30: 743BΔ*spo0A**11	Evolved strain from wild **ZQW10**	This study
ZQW31: 743BΔ*spo0A**11(pXY1-P_{thl}-*spo0A*)	743BΔ*spo0A**11/pXY1-P_{thl}-*spo0A*	This study
ZQW33: 743BΔ*spo0A**11(pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*)	743BΔ*spo0A**11/pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*	This study
ZQW35: 743BΔ*spo0A**11(pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*)	743BΔ*spo0A**11/pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*	This study
Plasmids		
pANS1	*Φ3TI* gene, p15A origin; $Spec^r$	(60)
pXY1-P_{thl}	Amp^r, MLS^r, pCB102 ORI, ColE1 origin, *Thl*	(61)

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	(*CA_c2873*) promoter region of *C. acetobutylicum*; *E. coli–C. cellulovorans* shuttle vector for expressing genes in *C. cellulovorans*	
pXY1-P_{thl}-*adhE1*	Derived from pXY1-P_{thl}, with *adhE1* (*CA_p0162*) gene from *C. acetobutylicum* overexpression	This study
pXY1-P_{thl}-*adhE2*	Derived from pXY1-P_{thl}, with *adhE2* (*CA_p0035*) gene from *C. acetobutylicum* overexpression	This study
pXY1-P_{thl}-*ctfAB-adc*	Derived from pXY1-P_{thl}, with *ctfAB-adc* cassette (*CA_p0163-0164-0165*) from *C. acetobutylicum* overexpression	This study
pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*	Derived from pXY1-P_{thl}, with *adhE1* gene and *ctfAB-adc* cassette from *C. acetobutylicum* overexpression	This study
pXY1-P_{thl}-*bukI*	Derived from pXY1-P_{thl}, with *bukI* (*CA_C3075*) gene from *C. acetobutylicum* overexpression	This study
pXY1-P_{thl}-*bukI*-P_{thl}-*adhE1*	Derived from pXY1-P_{thl}, with *bukI* and *adhE1* gene from *C. acetobutylicum* overexpression	This study
pWJ1-*spo0A*	Derived from pWJ1(62) for intron insertion in *C. cellulovorans spo0A* gene(*Clocel_1943*) at 586/587 nt	(6)
pXY1-P_{thl}-*spo0A*	Derived from pXY1-P_{thl}, with *C. cellulovorans spo0A* gene expression (complementation)	This study
pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*	Derived from pXY1-P_{thl}, with *adhE1* gene from *C. acetobutylicum* overexpression and *C. cellulovorans spo0A* gene complementation	This study
pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*	Derived from pXY1-P_{thl}, with *adhE1* gene and *ctfAB-adc* cassette from *C. acetobutylicum* overexpression, and *C. cellulovorans spo0A* gene complementation	This study

643 ATCC, American Type Culture Collection, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; Spe^r,
644 spectinomycin resistance; Amp^r, ampicillin resistance; MLS^r, macrolide–lincosamide–streptogramin resistance; pCB102 ORI,
645 Gram-positive origin of replication from *Clostridium butyricum*; Thl, thiolase.
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649 **TABLE 2 Primers used in this study.**

Primer name	Sequence (5'-3')	Description
EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	Targetron primer[a]
spo0A-586|587a -IBS	CCGCTCGAGATAATTATCCTTACTTTTCTTACAGGTG CGCCCAGATAGGGTG	*spo0A* Targetron primer[a]
spo0A-586|587a -EBS1d	AGATTGTACAAATGTGGTGATAACAGATAAGTCTTA CAGCTTAACTTACCTTTCTTTGT	*spo0A* Targetron primer[a]
spo0A-586|587a -EBS2	TGAACGCAAGTTTCTAATTTCGGTTAAAAGTCGATA GAGGAAAGTGTCT	*spo0A* Targetron primer[a]
spo0A_447-467	CGTATGAATCTAAGCCAGTAG	Forward primer inside *spo0A* from 447 to 467 base, Ch(intron-sF)
spo0A_743-764	TTGGTTTACCTTTTGATGTATG	Revise primer inside *spo0A* from 743 to 764 base, Ch(intron-sR)
spo0A F	TAGGAGGTTAGTTAGAGGATCCCCATGGACAATTC AAAAATAAGTG	Forward primer for gene *Clocel_1943* clone from 743B chromosome and assembly in plasmid pXY1-P_{thl}-*spo0A*
spo0A R	CGACGGCCAGTGAATTCCCTTAACTTACTTTGTTTT TAAGTC	Reverse primer for gene *Clocel_1943* clone from 743B chromosome and assembly in plasmid pXY1-P_{thl}-*spo0A*
adhE1 F	AAATTTAGGAGGTTAGTTAGAGGATCCCCATGAAA GTCACAACAGTAAAGGAATTAGAT	Forward primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*adhE1*
adhE1 R	GACGTTGTAAAACGACGGCCAGTGAATTCCCTTAA GGTTGTTTTTTAAAACAATTTATA	Reverse primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*adhE1*
adhE2 F	ATTTAGGAGGTTAGTTAGAGGATCCCCATGAAAGTT ACAAATCAAAAAGAACTAAAACA	Forward primer for gene *adhE2* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*adhE2*
adhE2 R	GTTGTAAAACGACGGCCAGTGAATTCCCTTAAAAT GATTTTATATAGATATCCTTAAGT	Reverse primer for gene *adhE2* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*adhE2*
bukI F	ATTTAGGAGGTTAGTTAGAGGATCCCCATGTATAGA TTACTAATAATCAATCCTGGCTC	Forward primer for gene *bukI* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *bukI*
bukI R	CGTTGTAAAACGACGGCCAGTGAATTCCCTTATTTG TATTCCTTAGCTTTTTCTTCTCC	Reverse primer for gene *bukI* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*bukI*
bukI F	ATTTAGGAGGTTAGTTAGAGGATCCCCATGTATAG ATTACTAATAATCAATCCTGGCTC	Forward primer for gene *bukI* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*bukI*-P_{thl}-*adhE1*
P_{thl}-adhE1-bukI R	TTATTATTTTTATCAATATATTTTGTTAAAAATTAT TTGTATTCCTTAGCTTTTTCTTC	Reverse primer for gene *bukI* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*bukI*-P_{thl}-

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		adhE1
bukI-P_{thl}-adhE1 F	GAAGAAAAAGCTAAGGAATACAAATAATTTTTAA CAAAATATATTGATAAAAATAATAA	Forward primer for cassette P_{thl}- *adhE1*clone from pXY1-P_{thl}-*adhE1* and assembly in plasmid pXY1-P_{thl}-*bukI*-P_{thl}- *adhE1*
adhE1 R	GACGTTGTAAAACGACGGCCAGTGAATTCCCTTAA GGTTGTTTTTTAAAACAATTTATA	Reverse primer for cassette P_{thl}- *adhE1*clone from pXY1-P_{thl}-*adhE1* and assembly in plasmid pXY1-P_{thl}-*bukI*-P_{thl}- *adhE1*
ctfAB-adc F	ATTTAGGAGGTTAGTTAGAGGATCCCCATGAACTCT AAAATAATTAGATTTGAAAATTT	Forward primer for cassette *ctfAB-adc* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*ctfAB-adc*
ctfAB-adc R	GACGTTGTAAAACGACGGCCAGTGAATTCCCTAAG TTTATATAAATCTATTATGCAGAA	Reverse primer for cassette *ctfAB-adc* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}-*ctfAB-adc*
adhE1 F	AAATTTAGGAGGTTAGTTAGAGGATCCCCATGAAA GTCACAACAGTAAAGGAATTAGAT	Forward primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*ctfAB-adc*
P_{thl}-ctfAB-dhE R	TATTATTTTTATCAATATATTTTGTTAAAAATTAAGGT TGTTTTTTAAAACAATTTATA	Reverse primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*ctfAB-adc*
adhE-ctfAB-adc F	TATAAATTGTTTTAAAAAACAACCTTAATTTTTAAC AAAATATATTGATAAAAATAATA	Forward primer for cassette P_{thl}-*ctfAB-adc* clone from pXY1-P_{thl}-*ctfAB-adc* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*
ctfAB-adc R	GACGTTGTAAAACGACGGCCAGTGAATTCCCTAAG TTTATATAAATCTATTATGCAGAA	Reverse primer for cassette P_{thl}-*ctfAB-adc* clone from pXY1-P_{thl}-*ctfAB-adc* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*
adhE1 F	AAATTTAGGAGGTTAGTTAGAGGATCCCCATGAAA GTCACAACAGTAAAGGAATTAGAT	Forward primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*spo0A*
P_{thl}-spo0A-adhE1 R	TATTATTTTTATCAATATATTTTGTTAAAAATTAAGGT TGTTTTTTAAAACAATTTATA	Reverse primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*spo0A*
adhE1-P_{thl}-spo0A F	TATAAATTGTTTTAAAAAACAACCTTAATTTTTAAC AAAATATATTGATAAAAATAATA	Forward primer for cassette P_{thl}-*spo0A* clone from pXY1-*spo0A* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*
spo0A R	CGACGGCCAGTGAATTCCCTTAACTTACTTTGTTTT TAAGTC	Reverse primer for cassette P_{thl}-*spo0A* clone from pXY1-*spo0A* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*spo0A*
adhE1 F	AAATTTAGGAGGTTAGTTAGAGGATCCCCATGAAA GTCACAACAGTAAAGGAATTAGAT	Forward primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*

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P_{thl}-ctfAB-adhE1 R	TATTATTTTTATCAATATATTTTGTTAAAAATTAAGGTTGTTTTTTAAAACAATTTATA	Reverse primer for gene *adhE1* clone from *C. acetobutylicum* genome and assembly in plasmid pXY1-P_{thl}- *adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*
adhE1-ctfAB-adc F	TATAAATTGTTTTAAAAAACAACCTTAATTTTTAACAAAATATATTGATAAAAATAATA	Forward primer for cassette P_{thl}-*ctfAB-adc* clone from pXY1-P_{thl}-*ctfAB-adc* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*
P_{thl}-spo0A-ctfAB-adc R	TATTATTTTTATCAATATATTTTGTTAAAAATAAGTTTATATAAATCTATTATGCAGAA	Reverse primer for cassette P_{thl}-*ctfAB-adc* clone from pXY1-P_{thl}-*ctfAB-adc* and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*
ctfAB-adc-P_{thl}-spo0A F	TTCTGCATAATAGATTTATATAAACTTATTTTTAACAAAATATATTGATAAAAATAATA	Forward primer for gene *Clocel_1943* clone from 743B chromosome and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*
spo0A R	CGACGGCCAGTGAATTCCCTTAACTTACTTTGTTTTTAAGTC	Reverse primer for gene *Clocel_1943* clone from 743B chromosome and assembly in plasmid pXY1-P_{thl}-*adhE1*-P_{thl}-*ctfAB-adc*-P_{thl}-*spo0A*

650 [a] Designed by on-line tools (www.clostron.com)

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653 **TABLE 3** Comparison of n-butanol production with single recombinant clostridia by CBP

Strains	Recombinant strategy	Substrates	Titer (g/L)	Productivity (g/L/h)	References
C. acetobutylicum ATCC 824	Overexpression optimization of chimeric cellulosomal operons	Crystalline cellulose	ND	ND	(15)
C. acetobutylicum ATCC 824	Grafting carrier modules at heterologous cellulases' N termini	Crystalline cellulose	ND	ND	(63)
C. acetobutylicum ATCC 824	Integrating synthetic cellulosomal operons into genome using allele-coupled exchange	Cellohexaose	ND	ND	(14)
C. acetobutylicum ATCC 824	Anchoring the recombinant cellulosome to the cell surface using the native sortase system	Untreated wheat straw	ND	ND	(13)
C. cellulolyticum ATCC 35319	Introducing CoA-dependent pathway	Crystalline cellulose	0.12	0.00025	(17)
C. cellulovorans DSM 743B	Overexpressing *adhE2* from *C. acetobutylicum ATCC 824*	Crystalline cellulose	1.42	0.0056	(28)
C. cellulovorans DSM 743B	Integrated metabolic and evolutionary engineering	Corn cobs (alkali extracted)	3.47	0.0413	This work

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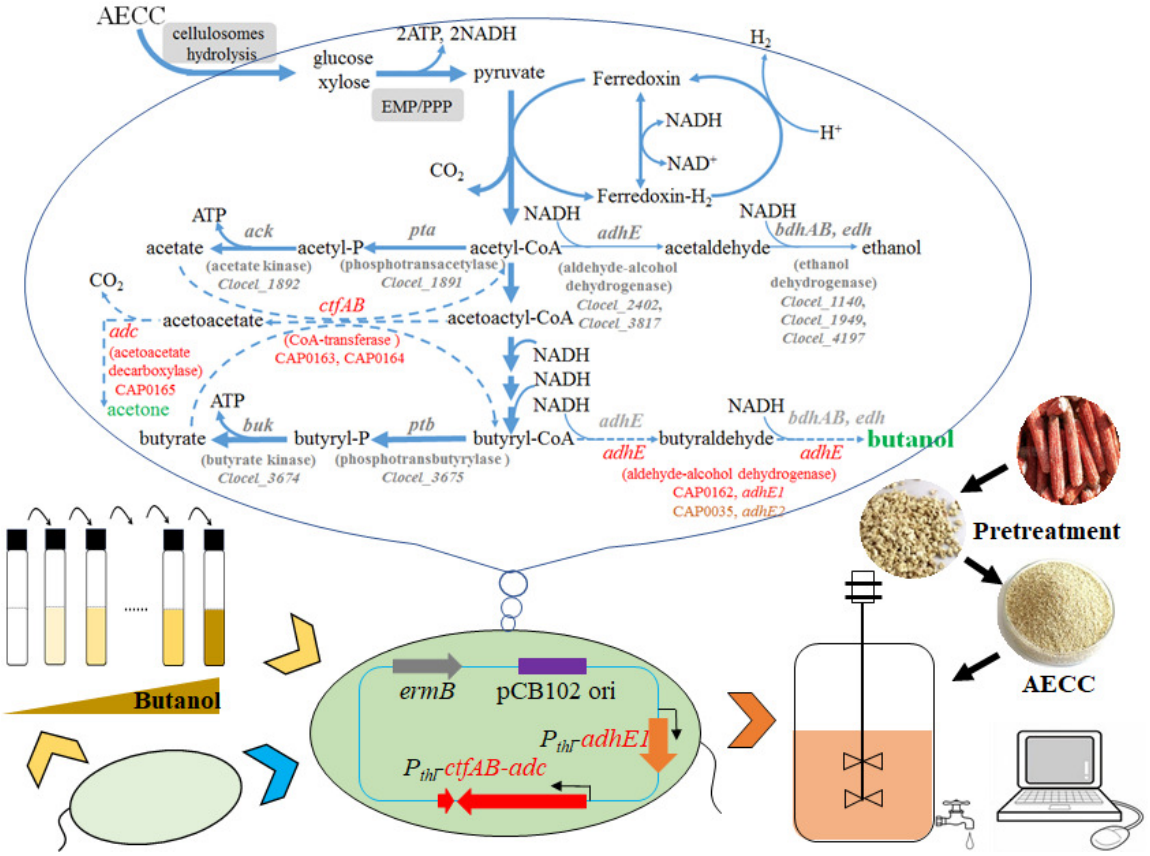
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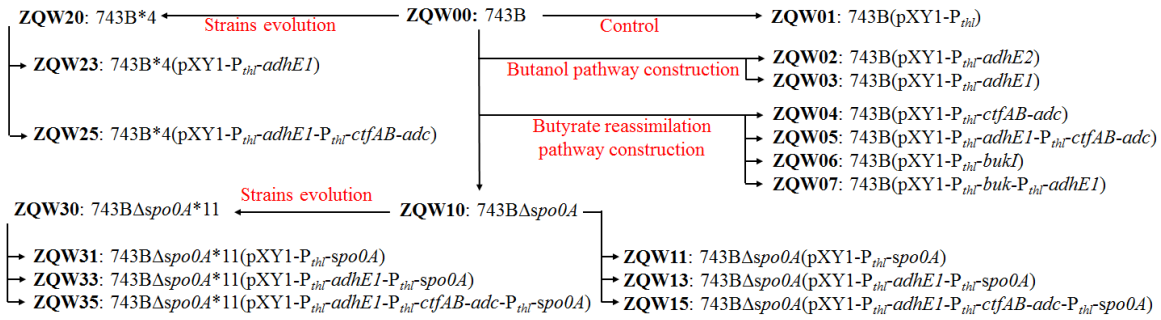
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FIG 1 Integrated metabolic and evolutionary engineering of *Clostridium cellulovorans* DSM 743B for n-butanol production from AECC by CBP. A CoA-dependent acetone-butanol-ethanol (ABE) pathway composed of *adhE1* and *ctfA-ctfB-adc* (red font) from *C. acetobutylicum* ATCC 824 was constructed in *C. cellulovorans*; In parallel, *C. cellulovorans* was grown in medium with a serial enrichment of n-butanol to evolve and reinforce its butanol tolerance; To maximize butanol production, metabolic and evolutionary engineering were combined, using the evolved strain as host for metabolic engineering.



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FIG 2 The dendrogram of genetically modified *C. cellulovorans* strains in this study



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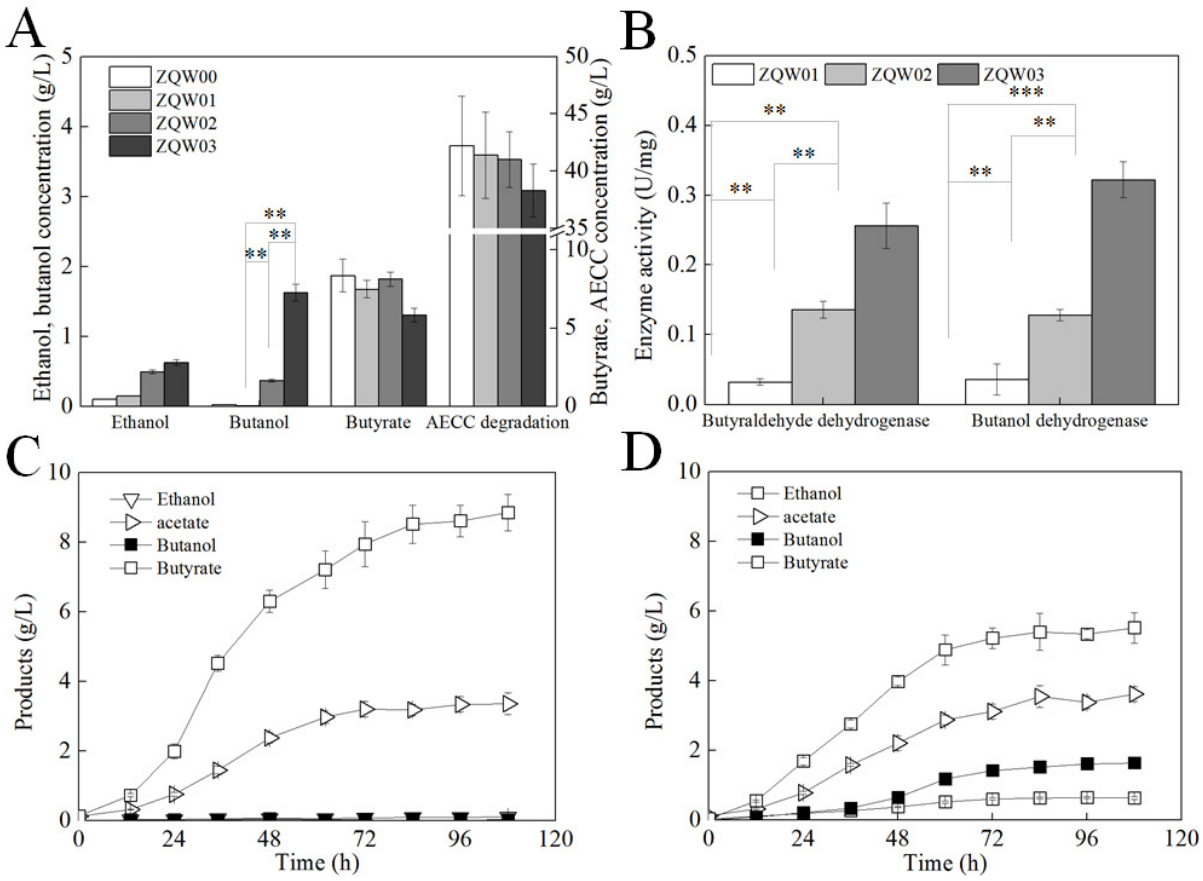
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FIG 3 Overexpression of *adhE1* enabled *C. cellulovorans* to produce more n-butanol than overexpressing *adhE2*. (A), effects of *adhE1* and *adhE2* introduction on the butanol production; (B), comparison of butyraldehyde dehydrogenase activity and butanol dehydrogenase activity between **ZQW02** and **ZQW03**; (C) and (D), fermentation profile of **ZQW01** and **ZQW03** respectively with AECC as sole carbon source. The data in (A) and (B) are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, $P>0.05$; t-test).



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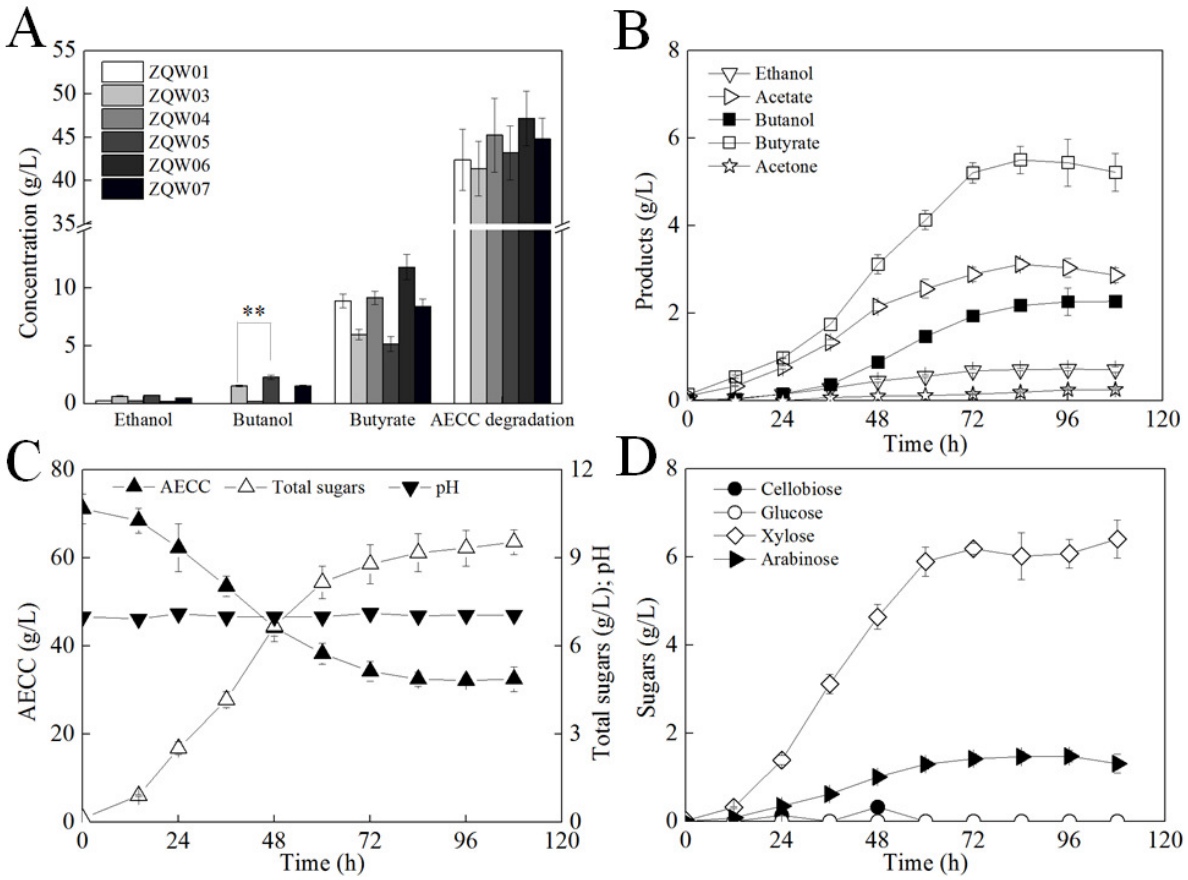
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FIG 4 Overexpressing *ctfAB-adc* in **ZQW03** drove n-butanol production by re-assimilating butyrate. (A), effects of *ctfAB-adc* and *bukI* introduction on the n-butanol production and butyrate reassimilation; (B), (C) and (D), batch fermentation profile of **ZQW05** with AECC as sole carbon source. The data in (A) are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, $P>0.05$; t-test).



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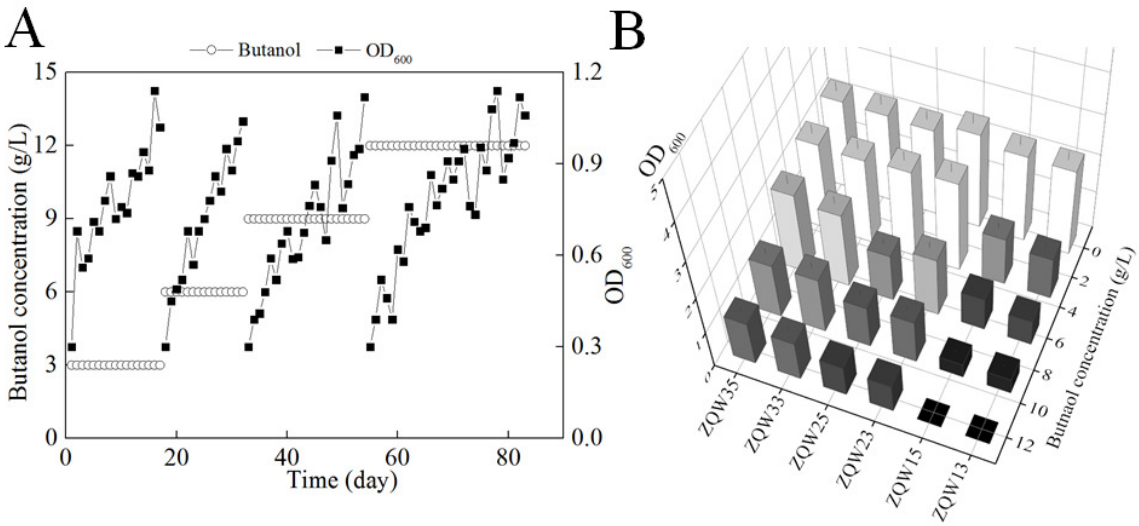
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FIG 5 Adaptive **l**aboratory evolution improved n-butanol tolerance of *C. cellulovorans*. (A), the evolution process of **ZQW10**. **ZQW10** was sub-cultured in media supplemented with stepwise enrichment of butanol (3, 6, 9, 12 g/L) to evolve and strengthen its n-butanol tolerance; (B) comparison of n-butanol tolerance of mutant strains derived from **ZQW10**, **ZQW20** and **ZQW30**. Due to evolution, **ZQW23**, **ZQW25**, **ZQW33** and **ZQW35** can tolerate higher concentration n-butanol than **ZQW13** and **ZQW15**. Besides, the n-butanol tolerance of **ZQW33** and **ZQW35** was superior to **ZQW23** and **ZQW25**.



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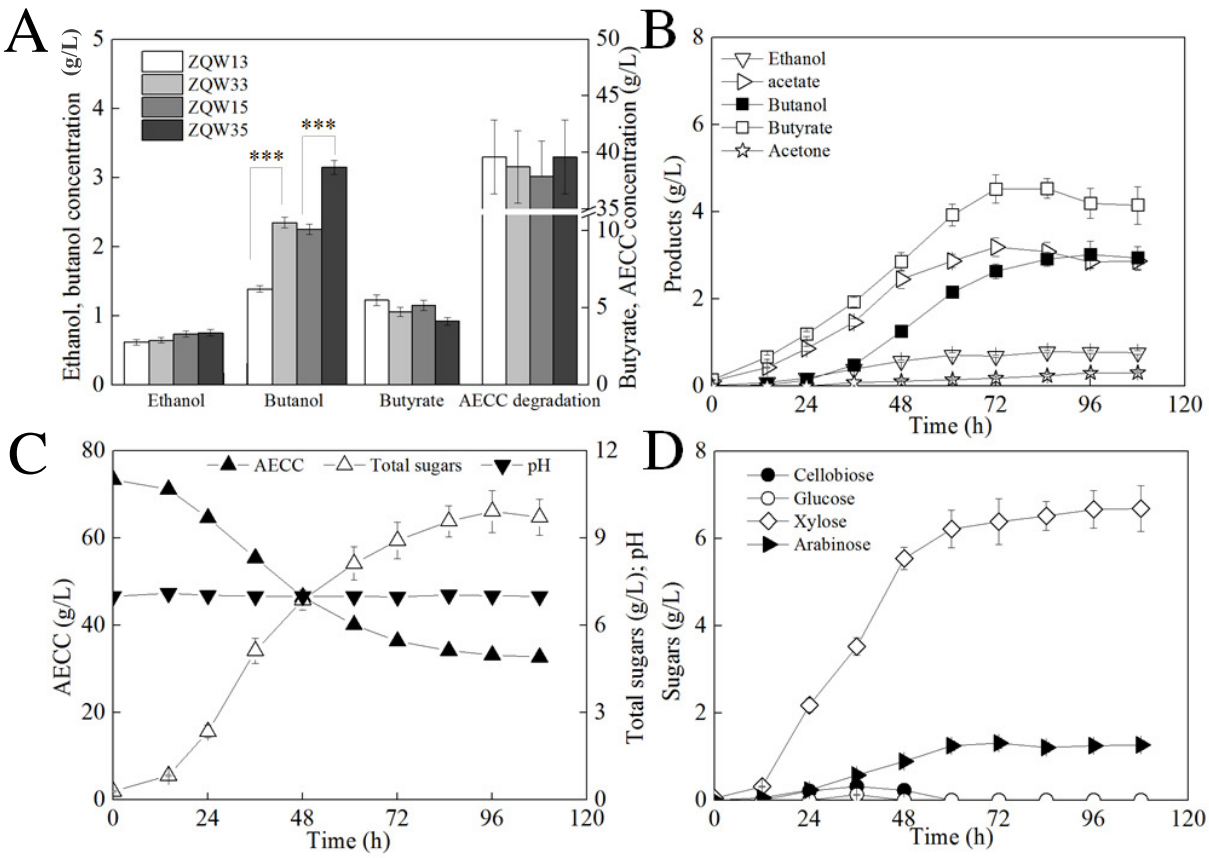
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FIG 6 Improved butanol tolerance led an enhancement in n-butanol production. (A), effects of laboratory adaptive evolution on the n-butanol production. Mutants **ZQW33** and **ZQW35** that derived from evolved strain **ZQW30**, produced much more n-butanol than the mutants **ZQW13** and **ZQW15** that derived from **ZQW10**.; (B), (C) and (D), metabolic profile of batch fermentation of **ZQW35** with AECC as sole carbon source. The data in (A) are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, $P>0.05$; t-test).



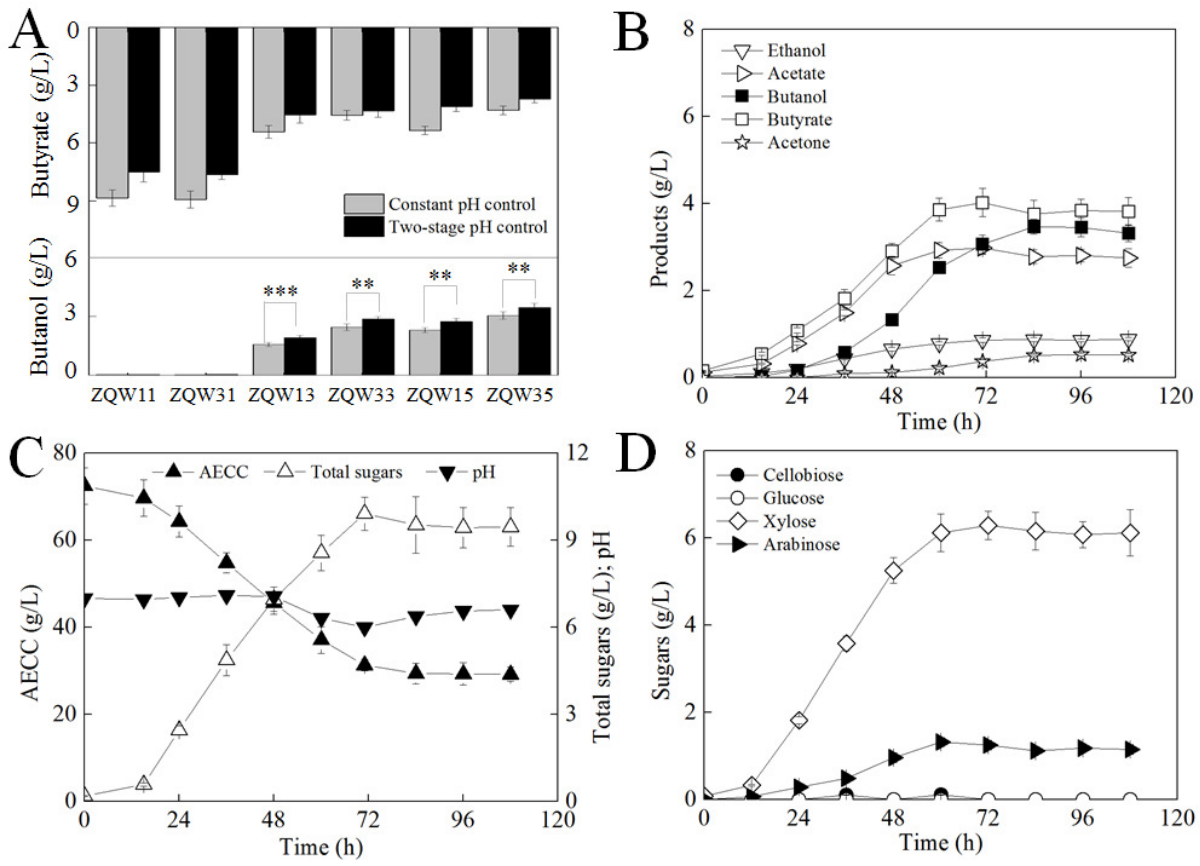
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FIG 7 Two-stage pH control strategy to improve n-butanol production further. (A), effects of two-stage pH control strategy on the n-butanol and butyrate production. Generally, two-stage pH control strategy; (B), (C) and (D), metabolic profile of batch fermentation of **ZQW35** using two-stage pH control strategy with AECC as sole carbon source. The data in (A) are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, $P>0.05$; t-test).



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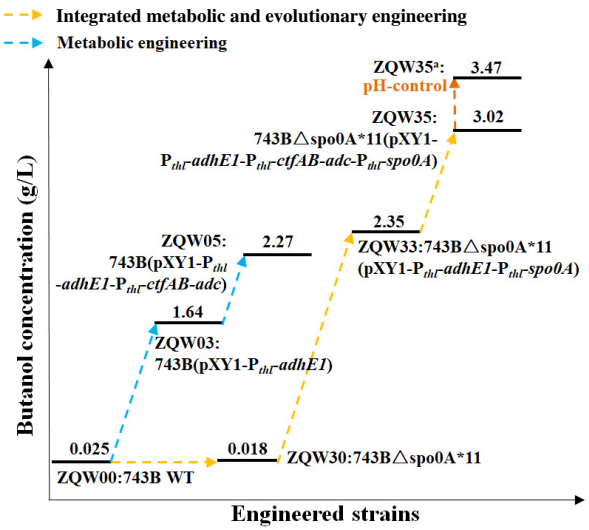
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FIG 8 Integrated metabolic and evolutionary engineering enhanced n-butanol production of *C. cellulovorans* from AECC.



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