Bioresource Technology 281 (2019) 217–225

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enhancement of acid re-assimilation and biosolvent production in *Clostridium saccharoperbutylacetonicum* through metabolic engineering for efficient biofuel production from lignocellulosic biomass

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ARTICLE INFO

Keywords:
Clostridium saccharoperbutylacetonicum N1-4
Acetone-butanol-ethanol (ABE)
Sol operon
Expression cassette EC
Acid re-assimilation
Metabolic engineering

ABSTRACT

In the clostridial acetone-butanol-ethanol (ABE) fermentation, the intermediate acetate and butyrate are re-assimilated for solvent production. Here, key genes in ABE pathways in *Clostridium saccharoperbutylacetonicum* N1-4 were overexpressed to enhance acid re-assimilation and solvent production. With the overexpression of *sol* operon, acid re-assimilation was enhanced, and ABE production was increased by 20%, with ethanol production increased by six times but almost no increase in butanol production. To further drive carbon flux for C4 metabolites and ultimate butanol production, key genes including *hbd, thl, crt* and *bcd* in butanol production pathway were further overexpressed. Compared to the control, butanol, acetone and total ABE production in the new strain was increased by 8%, 18%, and 12.4%, respectively. Finally, simultaneous saccharification and fermentation was carried out using acetate-pretreated switchgrass. 15.4 g/L total ABE (with a yield of 0.31 g/g) was produced in both engineered strains, which was significantly higher than the control.

1. Introduction

Lignocellulosic biomass is considered as the most promising long-term feedstock for the production of biofuels (Bhatia et al., 2017; Ezeji et al., 2007). The biomass typically needs to be pretreated prior to subsequent enzymatic hydrolysis to monomeric sugars and fermentation to biofuels. Pretreatment, however, generates a wide range of toxic compounds from the degradation of carbohydrates, lignin and extractives, which may significantly inhibit the subsequent microbial fermentation. The most common fermentation inhibitors in the hydrolysates include furan derivatives (furfural and 5-hydroxymethylfurfural (HMF)), phenolic compounds (such as coumaric acid, ferulic acid, syringaldehyde, and vanillin), and weak acids (mainly acetic acid and formic acid) (Jönsson and Martín, 2016). Among these degradation compounds, acetic acid, resulted from the hydrolysis of acetyl groups of hemicellulose, has been known to be the most prevalent organic acid accumulated in the hydrolysates of lignocellulosic biomass and is a severe inhibitor for various microbial fermentation processes, such as the ethanol fermentation with yeast (Wei et al., 2015).

Bio-butanol produced from renewable carbon sources through the clostridial acetone-butanol-ethanol (ABE) fermentation is of great interest, because it not only can be used as a renewable fuel that has various advantages over ethanol, but also has vast applications as a chemical feedstock in many industries (Jang et al., 2012; Zhou et al., 2014). The ABE fermentation was successfully operated in the industrial scale for biosolvent production in the early half of 20th century, but it gave way to chemical solvent synthesis from petroleum for economic reasons (Jones and Woods, 1986). Recently, the ABE fermentation received revived attention because of the high price and limited availability of petroleum oil and the surplus of waste lignocellulosic biomass materials that can be utilized as inexpensive fermentation feedstocks (Bhatia et al., 2017; Qureshi et al., 2013). Among the well-known solventogenic clostridial strains (from species of *Clostridium acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* and *C. saccharobutylicum*) that can be used for efficient ABE production, *C. saccharoperbutylacetonicum* N1-4 (ATCC 27021) can naturally produce very high levels of solvents and possesses various advantageous features (Tiam mun et al., 1995; Zhang et al., 2018b).

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https://doi.org/10.1016/j.biortech.2019.02.096
Received 7 January 2019; Received in revised form 19 February 2019; Accepted 20 February 2019
Available online 23 February 2019
0960-8524/

Table 1
Strains and plasmids used in this study.

Strains	Description and relevant characteristics	Sources
E. coli ER2523 (NEB express)	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10–TetS)2 [dcm] R(zgb-210::Tn10–TetS) endA1 Δ(mcrCmrr) 114::IS10*	New England Biolabs
C. saccharoperbutylacetonicum		
N1-4	DSM 14,923 (=ATCC 27021), wild type strain	DSM
JZ100	N1-4 harboring pJZ100	This work
PW2	N1-4 harboring pPW2	This work
PW3	N1-4 harboring pPW3	This work
PW4	N1-4 harboring pPW4	This work
Plasmids		
pTJ1	CAK1 ori, Amp^r, Erm^r	(Wang et al., 2013)
pJZ100	pTJ1 derivate; for gene overexpression under the control of the thiolase promoter (P_{thl}) from *C. saccharoperbutylacetonicum* N1-4	(Zhang et al., 2018b)
pSH7	pTJ1 derivative; for overexpression of cassette EC (*thl-hbd-crt-bcd*) under the thiolase promoter (P_{thl}) from *C. beijerinckii* NCIMB 8052	(Wang et al., 2017b)
pPW1	pSH7 derivative; containing additional P_{thl}-*Xho*I-T_{thl}; the thiolase promoter (P*thl*) was from *C. saccharoperbutylacetonicum* N1-4	This work
pPW2	pJZ100 derivative; containing additional *sol* operon *(ald-ctfA-ctfB-adc)*	This work
pPW3	pPW1 derivative; containing additional *sol* operon *(ald-ctfA-ctfB-adc)*	This work
pPW4	pSH7 derivative; containing additional P_{fdx}-*sol* operon	This work

Generally, ABE fermentation is a unique bi-phasic process. At the first phase (acidogenesis), carbohydrate carbon sources are degraded into acids (mostly acetic and butyric acids), while at the second phase (solventogenesis), the acids generated from the first phase are re-assimilated and converted into solvents along with the consumption of additional carbohydrates (Jones and Woods, 1986). In this sense, acetic acid (and butyric acid) is a substrate rather than an inhibitor for biobutanol production. Actually, the supplementation of additional acetate in chemically defined fermentation medium was found to increase and stabilize solvent production by various solventogenic clostridial strains (Chen and Blaschek, 1999a,b; Cho et al., 2012; Colin et al., 2001; Hüsemann and Papoutsakis, 1990). However, such effects have not been previously systematically investigated in *C. saccharoperbutylacetonicum* N1-4. Furthermore, little work has been done to develop robust *C. saccharoperbutylacetonicum* strains for enhanced acid re-assimilation and elevated solvent production.

In *C. saccharoperbutylacetonicum* N1-4, phosphotransacetylase (*pta*) and acetate kinase (*ack*) are responsible for the acetic acid production from acetyl-CoA and phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) are for the butyric acid production from butyl-CoA. Solventogenic genes are organized in a polycistronic solvent-producing *sol* operon consisting genes encoding NAD-dependent aldehyde dehydrogenase (*ald*; CSPA_RS27680), butyrate-acetoacetate CoA transferase subunits A/B (*ctfA/ctfB*; CSPA_RS27685/CSPA_RS27690), and acetoacetate decarboxylase (*adc*; CSPA_RS27695), among which *ctfA/ctfB* are genes primarily responsible for the acid re-assimilation (Kosaka et al., 2007). Along with the re-assimilation of acids, acetoacetate is produced followed by being transformed to acetone through the catalysis by *adc*. The cassette EC, including thiolase (*thl* CSPA_RS03020), β-hydroxybutyryl-CoA dehydrogenase (*hbd* CSPA_RS02150), crotonase (*crt* CSPA_RS2130), and butyryl-CoA dehydrogenase (*bcd* CSPA_RS2150), are responsible for the conversion of acetyl-CoA to butyryl-CoA (Hou et al., 2013). The final end products ethanol and butanol are produced under the action of the aldehyde dehydrogenase and alcohol dehydrogenase (encoded by *adhE*).

To improve alcohol titers and butanol selectivity, metabolic flux in the solventogenic biosynthesis and flow of carbon pathways have previously been enhanced through metabolic engineering in solventogenic clostridial strains. In *C. acetobutylicum*, the butanol reached an extremely high productivity ($2.64\ g\ L^{-1}\ h^{-1}$) in a long-term fermentation through overexpressing the *thl, ctfA/B* and *adhE1* genes as well as knocking out the *pta* and *buk* genes (Lee et al., 2016). By expressing the *sol* operon, optimizing the promoter of *aad* and co-expressing *thl*, total alcohol titers and butanol selectivity have been significantly increased (Sillers et al., 2009; Tummala et al., 2003). Hou et al. overexpressed the cassette EC (*thl, hbd*, *crt* and *bcd*) as well as the *adhE* and *ctfAB* genes from *sol* operon in *C. acetobutylicum*, resulting in 18.9 g/L of final butanol titer and 0.71 mol of butanol yield per mol of glucose consumed in a batch fermentation. Recently, the overexpression of *sol* operon in *C. saccharoperbutylacetonicum* strain N1-4 increased ethanol production by 400% with enhanced acid re-assimilation, and the overexpression of EC increased the butanol production (by 13.7%) and selectivity (by 73.7%) (Wang et al., 2017b).

For the biomass pretreatment, a method using acetic acid as the treatment reagent has recently been developed and the biomass hydrolysates has been used for ABE production by taking advantage of the unique acid re-assimilation capability of the solventogenic clostridia (Wang et al., 2019; Zhang et al., 2018a). However, in such a process, since elevated level of acetate is generated in the biomass hydrolysates, it will result in incomplete acetate re-assimilation and potential inhibition for cell growth. Therefore, in this study, the objective was to develop a robust strain with enhanced acid re-assimilation capability through metabolic engineering, to boost biosolvent production from acetic-acid-pretreated biomass. Various key genes related to acid re-assimilation and alcohol biosynthesis pathways including the *sol* operon (*ald-ctfA-ctfB-adc*) and cassette EC (*thl-hyd-crt-bcd*) were overexpressed in *C. saccharoperbutylacetonicum* N1-4. Fermentation resulted demonstrated that the engineered strain had reinforced capability for acid re-assimilation and solvent production and could efficiently convert the acetic-acid-pretreated biomass into ABE. Using the engineered strain, comprehensive utilization of lignocellulosic biomass for efficient biofuel production could be achieved.

2. Materials and methods

2.1. Strains and growth conditions

All bacterial strains used in this study are listed in Table 1. NEB® Express Competent *E. coli* ER2523 (New England Biolabs Inc., Ipswich, MA) was used for routine DNA cloning and vector maintenance. It was grown aerobically at 37 °C in the Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin (Amp) as needed. *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923, = ATCC 27021) was obtained from DSMZ (Braunschweig, Germany) and grown anaerobically at 35 °C in the tryptone-glucose-yeast extract (TGY) medium containing 30 g/l of tryptone, 20 g/l of glucose, 10 g/l of yeast extract, and 1 g/l of L-cysteine (Yao et al., 2017). 30 μg/ml of clarithromycin (Cla) was supplemented as needed for *C. saccharoperbutylacetonicum* mutant selection and cultivation.

Table 2
Primers used in this study.

Primers	Sequence (5′→3′)
YW32	GTTTTCCCAGTCACGACGTT
YW33	TTGCTGCTCATGCAGATGAT
YW1075	TCTATAAAATTTTAGGAGGTCAAACATGATTAAAGACACGCTAGTTTCTATAA
YW1076	TATCATAGTAACCTTTTTAAATCTTAATTTATATTATTTAAGGGAAAGATAATCATGTACAACC
YW2459	AAAGTTACTGTAGTTAGTATGGGACTTC
YW2460	AACAACTGGTATTAGTAATACTAAAACTGA
YW2491	AACCATCACACTGGCGGCCGTTTAAATATTATTATATGTGAGAAAAAATAAATTTG
YW2492	CTTTAATCATCTAGAACACCTCCTAATAAATTG
YW2493	GGTGTTCTAGATGATTAAAGACACGCTAG
YW2494	TTGGGCCCTCTAGATGCATGTTATTTAAGGGAAAGATAATCATG

2.2. Plasmid construction

The plasmids and primers used in this study were listed in Tables 1 and 2, respectively. All DNA primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). All cloning PCR was performed using the high-fidelity DNA polymerases, Phusion (New England BioLabs Inc., Ipswich, MA), PrimeSTAR (Takara Bio USA, Inc., Mountain View, CA), or Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China). The plasmids pTJ1 (Wang et al., 2016), pJZ100 (Zhang et al., 2018b) and pSH7 (Wang et al., 2017b) were used as mother vectors for the recombinant plasmid construction. The plasmid pSH7 containing the expression cassette EC (*thl-hyd-crt-bcd*) from *C. saccharoperbutylacetonicum* N1-4 has been previously derived from pTJ1 (Wang et al., 2017b). The plasmid pJZ100 has been previously derived from pTJ1 containing the *thiolase* promoter (P_{thl}) and terminator (T_{thl}) from *C. saccharoperbutylacetonicum* N1-4, with two *Bse*RI restriction enzyme sites in the middle between P_{thl} and T_{thl} (Zhang et al., 2018b). On the other hand, the same P_{thl}-T_{thl} fragment was inserted into the *Xho*I site of pSH7 through Gibson Assembly (NEBuilder® HiFi DNA Assembly Master Mix, New England Biolabs Inc., Ipswich, MA), generating pPW1. The *sol* operon including *ald, ctfA, ctfB* and *adc* was amplified from *C. saccharoperbutylacetonicum* N1-4 using primers YW1075 and YW1076, followed by being inserted into the *Bse*RI sites of pJZ100 and pPW1, generating pPW2 and pPW3, respectively. To obtain *sol* operon with the ferredoxin promoter (P_{fdx}), the two fragments of P_{fdx} and *sol* operon were amplified first from *C. saccharoperbutylacetonicum* N1-4 with primer pairs of YW2491/YW2492 and of YW2493/YW2494, respectively. Then the desirable P_{fdx} - *sol* fragment was generated through SOE-PCR with primers YW2491 and YW2494, followed by being inserted into the *Xho*I site of pSH7, generating pPW4. All the plasmid constructs were verified through Sanger sequencing performed by ACGT, Inc. (Wheeling, IL).

2.3. DNA transformation and mutant verification

The transformation of *C. saccharoperbutylacetonicum* N1-4 was carried out using electroporation following the protocol as previously described (Wang et al., 2017a). Briefly, *C. saccharoperbutylacetonicum* N1-4 was cultivated anaerobically at 35 °C in TGY medium until the optical density at 600 nm (OD_{600}) reached 0.8–1.0. The cells were harvested immediately through centrifugation at 4200*g* at 25 °C for 10 min. The cell pellets were washed once with SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, and 5 mM sodium phosphate, pH 6.5) with the same volume as the original volume of the bacterial culture at room temperature and then re-suspended in 1/20 vol of SMP buffer, obtaining the competent cells. Immediately, 1.0 μg of plasmid DNA was mixed with 400 μl of competent cells and transferred into a pre-cooled 0.2 cm electroporation cuvette. The whole mixture within the cuvette was then incubated in ice for 20 min. The whole process was carried out by transferring the cell culture in and out the anaerobic chamber to avoid exposing the cells to oxygen (the centrifugation needed to be performed outside of the chamber). A Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA) connected to the anaerobic chamber was used to deliver the electronic pulse with the following conditions: 1000 V of voltage, 25 μF of capacitance and 300 Ω of resistance. Afterwards, the cells were transferred into 1.6 ml of TGY and incubated at 35 °C for 2–4 h for recovery. The recovered cells were plated onto pre-warmed TGY plates containing clarithromycin and incubated anaerobically at 35 °C. After 1–2 days, Cla-resistant colonies would appear and were picked for colony PCR (cPCR) to confirm the presence of plasmid using primers YW32 and YW33 (for pPW2) or YW2459 and YW2460 (for pPW3 or pPW4). The generated recombinant strains were named based on the harbored plasmid as *C. saccharoperbutylacetonicum* PW2, *C. saccharoperbutylacetonicum* PW3, *C. saccharoperbutylacetonicum* PW4, and *C. saccharoperbutylacetonicum* JZ100 (as a control strain), respectively.

2.4. Fermentation

Batch fermentation was carried out with a model solution containing 80 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone and filter-sterilized P2 or modified P2 (MP2) medium. The P2 medium contains (in g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; CH_3COONH_4, 2.2; $MgSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.01; NaCl, 0.01; p-aminobenzoic acid, 0.001; thiamine-HCl, 0.001; and biotin, 0.00001. The MP2 medium is the same as P2 medium except that 2 g/L $(NH_4)_2SO_4$ was used in MP2 to replace the 2.2 g/L of CH_3COONH_4 in P2.

To investigate the effect of acetate on the solvent production in various strains, the fermentation was performed in 250 ml serum bottles with a working volume of 100 ml. Before starting the fermentation, the stock solution of glucose, and that of mixed yeast extract and tryptone were both set to pH 6.8, sparged with N_2 for 10 min and autoclaved at 121 °C for 20 min. After being cooled down, they were put into the anaerobic chamber. Along with filter-sterilized MP2 or P2 stock solutions with different concentration of sodium acetate (when necessary), all the necessary components were mixed together to reach the designated composition for each fermentation. This included 80 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone, and various concentrations (0–12.6 g/L; there is around 1.6 g/L acetate in the P2 medium) of acetate. Then, active growing preculture (at OD_{600} of 0.8–1.0 grown in TGY) was inoculated into the fermentation with an inoculum ratio of 5% (v/v). Then all the bottles were put into a shaker incubator and the fermentation was carried out at 30 °C with 150 rpm agitation without pH control. All the serum bottles were kept sealed during the fermentation to maintain the anaerobic condition. All fermentations were performed in triplicates.

Large-scale batch fermentation was performed in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 L. Model solution of the same composition as described above for the fermentation in serum bottles was added into the reactor and then autoclaved. Oxygen-free nitrogen was flushed through the broth starting overnight or at least several hours before the

inoculation (until the fermentation culture initiated its own gas production).

The cell culture was propagated anaerobically in the TGY medium until the OD_{600} reached ~0.8. Then the culture was inoculated into the reactor at 5% (vol/vol) inoculum ratio to start the fermentation. The temperature was controlled at 30 ± 1 °C and the agitation was maintained at 55 rpm. The pH was controlled > 5.0 throughout the fermentation by adding 6 M NaOH. Samples were taken throughout the fermentation to monitor the cell density, sugar consumption, and endproduct production. Each fermentation was conducted in duplicate. The pH profile was automatically recorded by the NBS BioCommand software (New Brunswick Scientific Co, Inc., Edison, NJ) in real time throughout the fermentation.

2.5. Analytical procedures

Cell growth was monitored by measuring OD_{600} with a Ultrospec 10 cell density meter (Amersham Biosciences Corp., Piscataway, NJ). An Agilent 1260 Infinity HPLC system (Agilent Technologies, CA) was used for the analyses of sugars and fermentation endproducts. The various compounds were separated with a Varian MetaCarb 87H Column (300 × 7.8 mm) along with a 50 × 4.6 mm MetaCarb 87H guard column (Agilent Technologies, CA) and then detected with the refractive index detector (RID). 0.005 N H_2SO_4 was used as the mobile phase at an isocratic flow rate of 0.6 ml/min, and the temperature of the column was maintained at 25 °C during the elution.

All the statistical analyses were performed using SAS® University Edition software (SAS Institute Inc, Cary, NC). Results with *p*-values below the conventional 5% threshold were regarded as significant.

3. Results and discussions

3.1. Effects of the sol operon overexpression on solvent production

As shown in Fig. 1, in the fermentation, the kinetics for cell growth and sugar consumption of *C. saccharoperbutylacetonicum* PW2 were similar to *C. saccharoperbutylacetonicum* JZ100. The pH profile of PW2 firstly decreased to around 5.5 and then increased back, and further decreased again after 20 h. However, the pH never reached a point < 5.0 in the whole process. The acetate level decreased from the very beginning of the fermentation and leveled off at 0.3 g/L from 36 h till the end of the fermentation. The peak level of butyrate production in PW2 was only about 1/3 of that in JZ100, and there was no detectable butyrate production at the end of the fermentation. These results about acetate and butyrate production indicated that PW2 had significantly enhanced acid re-assimilation capability because of the overexpression of *sol* operon. Significantly higher ethanol had been produced in PW2 (4.9 g/L vs. 0.7 g/L in JZ100). Also, acetone and butanol production in PW2 had been improved by 19.2% and 3.5% respectively compared to the control. Overall, the total solvent production was improved to 31.4 g/L (compared to 25.1 g/L in JZ100), which was the highest among all the strains constructed in this study (Fig. 1I). It was not surprising that the overexpression of *ctfA/B* and *adc* had led to significantly increased acetone production. The *ald* gene encodes an NAD-dependent aldehyde dehydrogenase; it has the activity for catalyzing the production of both acetaldehyde and butyraldehyde (and thus enhances the further production of ethanol and butanol) (Herman et al., 2017). The overexpression of *ald* led to six times more ethanol production in PW2 than in JZ100, however, the butanol production in PW2 was only slightly increased. This might be because *ald* within the *sol* operon has higher specificity for ethanol production rather than for butanol production (Kosaka et al., 2007). On the other hand, it is more 'cost-efficient' (less reducing power is needed) for the cell to convert acetyl-CoA to ethanol rather than to butyryl-CoA and further to butanol.

Previously, *sol* operon had been overexpressed in *C. saccharoperbutylacetonicum* driven by the $P_{thl\text{-}Cbe}$ from *C. beijerinckii* NCIMB 8052, which led to slightly decreased butanol production and marginally increased ABE production by the mutant pSH1 strain compared to the control strain (Wang et al., 2017b). In this study, the native P_{thl} was employed for the overexpression of *sol* operon and resulted in enhanced butanol (PW2: 17.7 g/L vs pSH1: 12.6 g/L) and much more elevated ABE production (PW2: 31.4 g/L vs pSH1: 24.3 g/L). This suggested that the native promoter enabled superior activity of the overexpressed genes, and thus is a preferable option for gene overexpression purpose.

3.2. Effects of combined overexpression of sol operon and EC on solvent production

Further, both *sol* operon and cassette EC were overexpressed in *C. saccharoperbutylacetonicum* to investigate whether such a strategy could have complementary and synergistic effects on the solvent production. As shown in Fig. 1, the constructed strain PW3 (with one P_{thl} to drive the *sol* operon expression, and another P_{thl} to drive the EC expression; Table 3) demonstrated delayed growth at the beginning of the fermentation when compared to JZ100. The sugar consumption was a little delayed than JZ100. The pH initially decreased to ~5.25 (and never reached a point < 5.0 as JZ100 did) and then increased back. For the acetate production, there was no noticeable increase from the beginning of the fermentation, and then decreased to lower levels after 12 h of the fermentation, and only 0.3 g/L acetate was detected at the end. The peak value of butyrate in PW3 was lower than in JZ100, and by the end of the fermentation, there was no butyrate left. These results indicated that the co-overexpression of *sol* operon in PW3 brought about an efficient acid re-assimilation. After the fermentation, there was 1.2 g/L ethanol produced, which as slightly higher than JZ100, but much lower than PW2. The acetone production was similar to that in PW2 and 17.8% higher than that in JZ100. The butanol production reached 18.4 g/L in PW3, which was the highest among all these strains (Table 3). In addition, 28.2 g/L total ABE was produced in PW3, which was 12.4% higher than that in JZ100, but 10.2% lower than that in PW2. Therefore, briefly, by co-expression of *sol* operon and EC in PW3, the combined benefits were indeed observed for enhanced acid re-assimilation and elevated butanol production.

Since P_{thl} was used for the overexpression of both the *sol* operon and EC in PW3, it was speculated that the competition for the same RNA polymerase could have impeded the expression of the target genes. In addition, this could also possibly negatively influence the native thiolase gene expression due to the promoter titration effects (Sillers et al., 2009). Therefore, it was hypothesized that the performance of the recombinant strain (particularly PW3) could be possibly further improved by overexpressing these two gene clusters (*sol* operon and *EC*) using two different strong constitutive promoters. The promoter of the ferredoxin gene (P_{fdx}) has been known as another constitutive strong promoter (Woods, 1995). Thus, *C. saccharoperbutylacetonicum* PW4 was constructed by overexpressing EC under P_{thl} and *sol* operon under P_{fdx}. As shown in Fig. 1 and Table 3, the phenotype for the cell growth, sugar consumption, and endproduct production in PW4 were all very similar to PW3. Actually, the production of acetone, ethanol and butanol in PW4 was all slightly decreased when compared to PW3. The final total solvent in PW4 was 6.7% lower than that in PW3. Such results were unexpected but reasonable. P_{fdx} is a very strong promoter; however, it might not be as strong as P_{thl}, and thus the solvent production in PW4 was not further increased (but decreased) as compared to PW3. These results also suggested that the promoter titration effect was possibly not a problem in PW3.

3.3. Effects of supplemented acetate on the fermentation with various recombinant strains

With the metabolic engineering efforts in this study, the acid re-

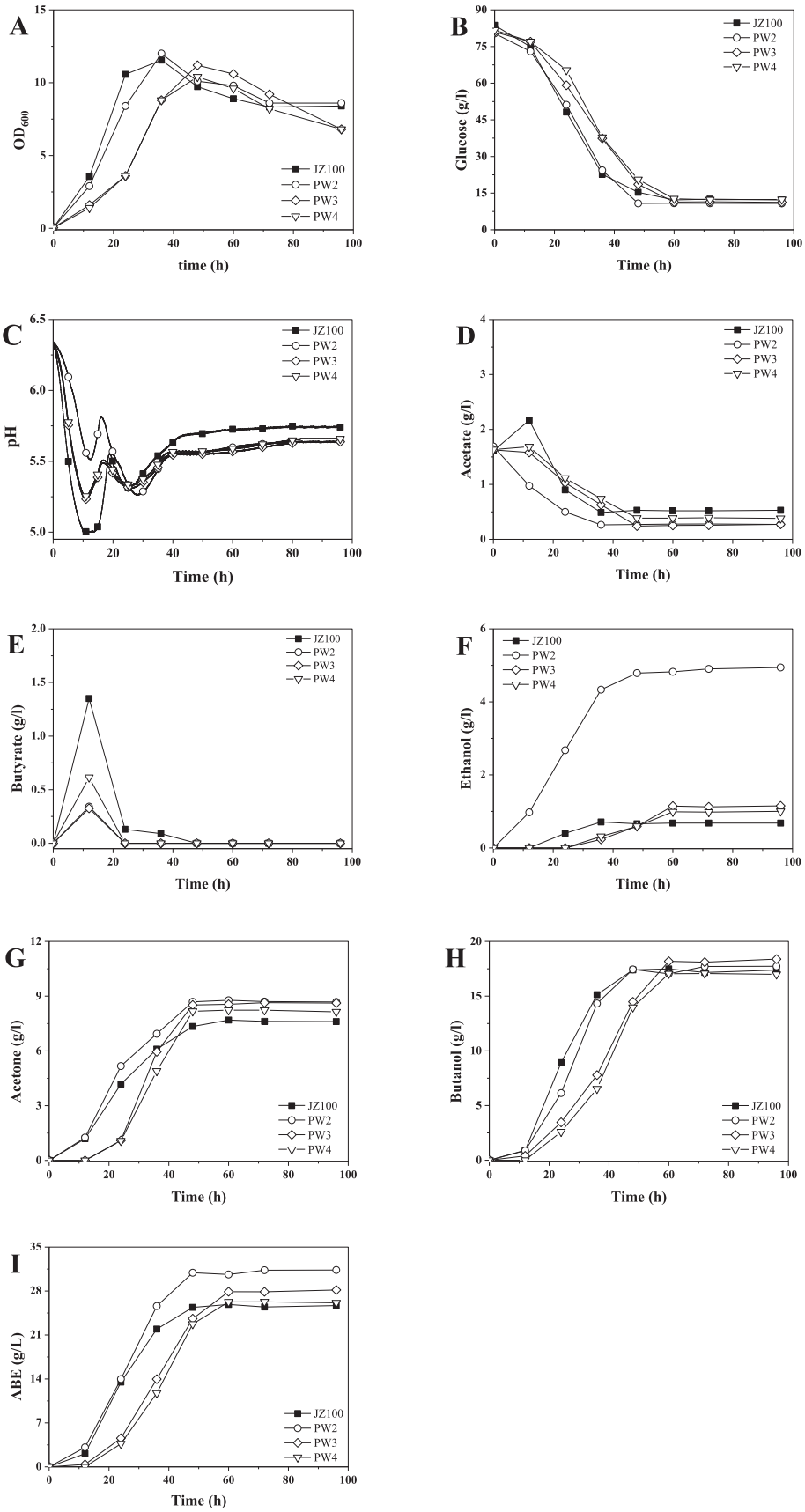

Fig. 1. Batch fermentation profiles of *C. saccharoperbutylacetonicum* mutant strains in the P2 medium. (A) Cell optical density; (B) Glucose consumption; (C) pH; (D) Acetate production; (E) Butyrate production; (F) Ethanol production; (G) Acetone production; (H) Butanol production; (I) Total ABE. Fermentation was carried out in replicates, with the results from one batch reported here as the representative.

Table 3
Summary of the fermentation results for *C. saccharoperbutylacetonicum* strains using the P2 model solution with 80 g/L glucose as carbon source.*

Strains				
Results	JZ100	PW2	PW3	PW4
Glucose consumed (g/L)	71.4 ± 1.0[a]	69.6 ± 1.5[a]	69.7 ± 1.2[a]	69.3 ± 1.1[a]
Acetone (g/L)	7.3 ± 0.6[a]	8.7 ± 0.5[a]	8.6 ± 0.4[a]	8.2 ± 0.4[a]
Ethanol (g/L)	0.7 ± 0.1[a]	4.9 ± 0.2[b]	1.2 ± 0.1[a]	1.0 ± 0.1[a]
Butanol (g/L)	17.1 ± 0.6[a]	17.7 ± 0.2[a]	18.4 ± 0.3[a]	17.0 ± 0.4[a]
Final solvents (g/L)	25.1 ± 0.8[a]	31.4 ± 0.7[b]	28.2 ± 1.1[ab]	26.3 ± 1.1[ab]
Final solvent yield (g/g)	0.35 ± 0.01[a]	0.45 ± 0.01[b]	0.41 ± 0.01[ab]	0.38 ± 0.02[ab]
Acetic acid (g/L)	0.5 ± 0.4[a]	0.3 ± 0.3[a]	0.3 ± 0.1[a]	0.4 ± 0.2[a]
Butyric acid (g/L)	0.1 ± 0.1[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]

* All results shown are the average value ± standard deviations from replicated experiments. Values with the same letter within the same row are not significantly different at the 0.05 level based on Tukey's HSD test.

assimilation capability in the host strain was attempted to be enhanced and thus solvent production could be elevated. In the lignocellulosic biomass hydrolysates (especially when acetate is used as the chemical reagent for the pretreatment), higher levels of acetate (than that already exists in the P2 medium) are expected (Wang et al., 2019). Therefore, the performance of various recombinant strains constructed in this study were further systematically evaluated when various concentrations of acetate were supplemented in the fermentation.

As shown in Table 4 and Fig. 2, when there is no acetate supplemented, the solvent production was low for all strains, indicating that appropriate amount of acetate is required for the buffering purpose for solvent production (Chen and Blaschek, 1999b). However, comparatively, all the engineered strain produced more solvent than the control strain, in the order of PW3 > PW2 > PW4 > JZ100. This indicated that the overexpression of *sol* operon is critical for the solvent production especially when there is no acetate is supplemented. Previously, it has been reported that when acetate was supplemented in the ABE fermentation with *C. beijerinckii* NCIMB 8052, the CoA transferase activity was elevated and thus led to enhanced solvent production. While when there was no acetate supplemented as the case here, the overexpression of *sol* operon could offset the inefficiency of *sol* operon activity in the wild type strain and thus significantly increased the solvent production. With the increase of supplemented acetate concentration, the production of acetone, ethanol, butanol as well as the total ABE in each strain generally increased and reached the maximum level when 4.6 g/L acetate was supplemented. With the further increase of the supplemented acetate concentration (from 5.6 to 8.6 g/L), the solvent production in JZ100 gradually decreased; however, the solvent production in the engineered strains kept at the similar level with some marginal decrease (as that when 4.6 g/L acetate was supplemented). This demonstrated that, with the overexpression of key genes in the solvent production pathway (especially with the overexpression of *sol* operon), the acid re-assimilation and solvent production was enhanced in the recombinant strains. From another perspective, it could be concluded that the tolerance to the high level fatty acids had been reinforced in the engineered strain compared to the control. However, when the supplemented acetate was increased to 12.6 g/L, the solvent production in all the strains has been remarkably decreased. It suggested that the high level of acetate under this condition is too toxic and thus the ABE production was inhibited even in strains with the overexpression of the ABE pathway genes. Under all these conditions, PW3 generally produced the highest levels of butanol, while PW2 produced the highest ethanol and thus highest total ABE (except for the condition when there is no acetate added) among all the strains (Fig. 2D).

3.4. ABE production from acetic-acid-treated switchgrass through simultaneous saccharification and fermentation (SSF)

An innovative acetic-acid-based biomass pretreatment method for enhanced biosolvent production has been previously developed (Wang et al., 2019). With acetic acid as the catalyst for biomass pretreatment, no additional chemical is introduced and thus saves cost and avoids the associated environmental pollution. In addition, generally with such a mild chemical reagent for biomass pretreatment, less amount of inhibitors will be generated compared to the conventional biomass pretreatment methods. Thus, the liquid prehydrolysates fraction (LPF) from the acetic-acid-based pretreatment (which is usually discarded during the regular biomass pretreatment process because of its low fermentability) can be used for the fermentation along with the solid cellulosic fraction (SCF) to provide additional carbon source. The supplemented acetic acid for the pretreatment (along with the acetic acid generated during the process) will be re-assimilated as additional carbon source for ABE production in the clostridial fermentation, thus leading to improved solvent yield. However, with the high level of acetate in the hydrolysates, the wild type strain for ABE fermentation cannot take up all the acetate for solvent production (Wang et al., 2019). It was hypothesized that the constructed recombinant strains in this study could work better for the fermentation with this substrate due to their enhanced acid re-assimilation capability. Based on the results with model solutions as described above, the PW2 and PW3 strains demonstrated the best potential for solvent production when high levels of fatty acid existed in the medium. In addition, 4.6 g/L of supplemented acetate in the medium was likely the most appropriate for the solvent production (Fig. 2). Therefore, switchgrass (a total of 100 ml slurry containing a total amount of 10 g dry biomass) was pretreated with 3 g/L acetic acid (which would generate around 4.6 g/L acetate in the hydrolysates) at 170 °C for 20 min as described previously (Wang et al., 2019). After processing, the LPF/SCF mixture was used for SSF with PW2 and PW3. As shown in Table 5, compared to the control JZ100, PW2 and PW3 had more efficient acid (both acetic and butyric acids) re-assimilation, and produced significantly higher levels of acetone and ethanol and slightly higher butanol, with 15.4 g/L total ABE (20% higher than JZ100) generated. The ABE yield was also ~20% higher in PW2 and PW3 than in JZ100. These results demonstrated that the engineered strains in this study could be used as robust platform for ABE production from lignocellulosic biomass processed with the tailored acetic-acid pretreatment approach.

4. Conclusions

Key genes in ABE pathways in *C. saccharoperbutylacetonicum* were overexpressed to enhance acid re-assimilation and solvent production. First, the *sol* operon was overexpressed, and acid re-assimilation was improved and ABE production reached 31.4 g/L. To further drive carbon flux from C2 to C4 metabolites for butanol production, the EC cassette was further overexpressed (besides *sol*), and the butanol production was increased by 8% than control. Finally, SSF was carried out using acetic-acid-pretreated switchgrass as feedstock. 15.4 g/L total ABE (with a yield of 0.31 g/g) was produced in engineered strains, which was significantly higher than the control.

Table 4
Summary of the fermentation results of *C. saccharoperbutylacetonicum* mutant strains with various concentrations of acetate supplemented.

	Initial acetate concentration (g/L)									
	Strains[a]	0	1.6	2.6	3.6	4.6	5.6	6.6	7.6	8.6	12.6
Glucose consumption (g/L)	JZ100	44.9 ± 1.1	70 ± 0.3	74.6 ± 0.3	75.7 ± 1.1	76.7 ± 0.9	76.6 ± 1.0	76.1 ± 0.9	72.6 ± 0.2	70.1 ± 0.1	42.7 ± 0.1
	PW2	47.4 ± 1.5	71.9 ± 0.9	75.9 ± 0.4	77.4 ± 0.2	78.5 ± 0.4	79.7 ± 0.5	79.7 ± 0.1	79.8 ± 0	79.7 ± 0.1	80 ± 0
	PW3	45.5 ± 0.1	69.6 ± 0.3	70.4 ± 0.3	76.6 ± 1	79.5 ± 1	79.1 ± 0.1	78.8 ± 0.8	79.7 ± 0.5	80 ± 0	80 ± 0
	PW4	45.3 ± 1.1	68.1 ± 0.3	70.6 ± 0.3	75.7 ± 1	78.8 ± 1	79.3 ± 1	79.5 ± 0.9	79.5 ± 0.7	79.6 ± 0.9	79 ± 0
Acetate re-assimilation (g/L)	JZ100	−0.4 ± 0.2	1.1 ± 0.1	1.7 ± 0	2.6 ± 0	3.2 ± 0	3.6 ± 0	4.1 ± 0.1	3 ± 0.4	3.4 ± 0.4	2.4 ± 0.1
	PW2	−0.1 ± 0.1	1.3 ± 0	2.4 ± 0	3.2 ± 0	3.9 ± 0	4.5 ± 0	4.8 ± 0.1	5.4 ± 0.2	5.7 ± 0.1	7.2 ± 0.2
	PW3	0 ± 0	1.1 ± 0	1.6 ± 0	2.3 ± 0	2.9 ± 0	3.2 ± 0	3.7 ± 0	4.6 ± 0	4.5 ± 0	6.6 ± 0.1
	PW4	0 ± 0	1.3 ± 0.2	1.6 ± 0	2.2 ± 0	2.7 ± 0	2.8 ± 0	2.8 ± 0.1	2.9 ± 0.1	3.6 ± 0.1	6.1 ± 0
Acetate (g/L)	JZ100	0.4 ± 0.2	0.6 ± 0.1	0.9 ± 0	1 ± 0	1.4 ± 0	2 ± 0	2.5 ± 0.1	4.6 ± 0.4	5.2 ± 0.4	10.2 ± 0.1
	PW2	0.1 ± 0.1	0.3 ± 0	0.2 ± 0	0.4 ± 0	0.7 ± 0	1.1 ± 0	1.8 ± 0.1	2.2 ± 0.2	2.9 ± 0.1	5.5 ± 0.2
	PW3	0 ± 0	0.5 ± 0	1 ± 0	1.3 ± 0	1.7 ± 0	2.4 ± 0	2.9 ± 0	3 ± 0	4.1 ± 0	6 ± 0.1
	PW4	0 ± 0	0.3 ± 0.2	1.1 ± 0	1.4 ± 0	1.9 ± 0	2.8 ± 0	3.8 ± 0.1	4.7 ± 0.1	5 ± 0.1	6.5 ± 0
Butyrate (g/L)	JZ100	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.1	1.3 ± 0.1	1.5 ± 0	1.2 ± 0	1.9 ± 0
	PW2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0.2
	PW3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.7 ± 0.8
	PW4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.7 ± 0.5
ABE yield (g/g)	JZ100	0.31 ± 0.02	0.31 ± 0.01	0.32 ± 0.01	0.33 ± 0.05	0.34 ± 0.03	0.32 ± 0.04	0.32 ± 0.04	0.32 ± 0	0.32 ± 0	0.36 ± 0.02
	PW2	0.37 ± 0.13	0.41 ± 0.09	0.41 ± 0.13	0.42 ± 0.11	0.43 ± 0.18	0.42 ± 0.14	0.42 ± 0.15	0.41 ± 0.09	0.42 ± 0.12	0.36 ± 0.13
	PW3	0.42 ± 0.19	0.41 ± 0.11	0.41 ± 0.15	0.4 ± 0.15	0.4 ± 0.03	0.4 ± 0.27	0.4 ± 0.15	0.4 ± 0.04	0.39 ± 0.07	0.34 ± 0.11
	PW4	0.36 ± 0.02	0.38 ± 0.01	0.39 ± 0.01	0.38 ± 0.05	0.38 ± 0.03	0.37 ± 0.04	0.38 ± 0.04	0.38 ± 0.51	0.38 ± 0.26	0.31 ± 0.32

[a] Cultures were grown in 100 ml MP2 medium with 80 g/L glucose and various levels of acetate (0–12.6 g/L acetate; in the form of 0–17.5 g/L sodium acetate). Samples were examined after the fermentation was run for 96 h. The reported values are mean ± standard deviation of triplicated samples.

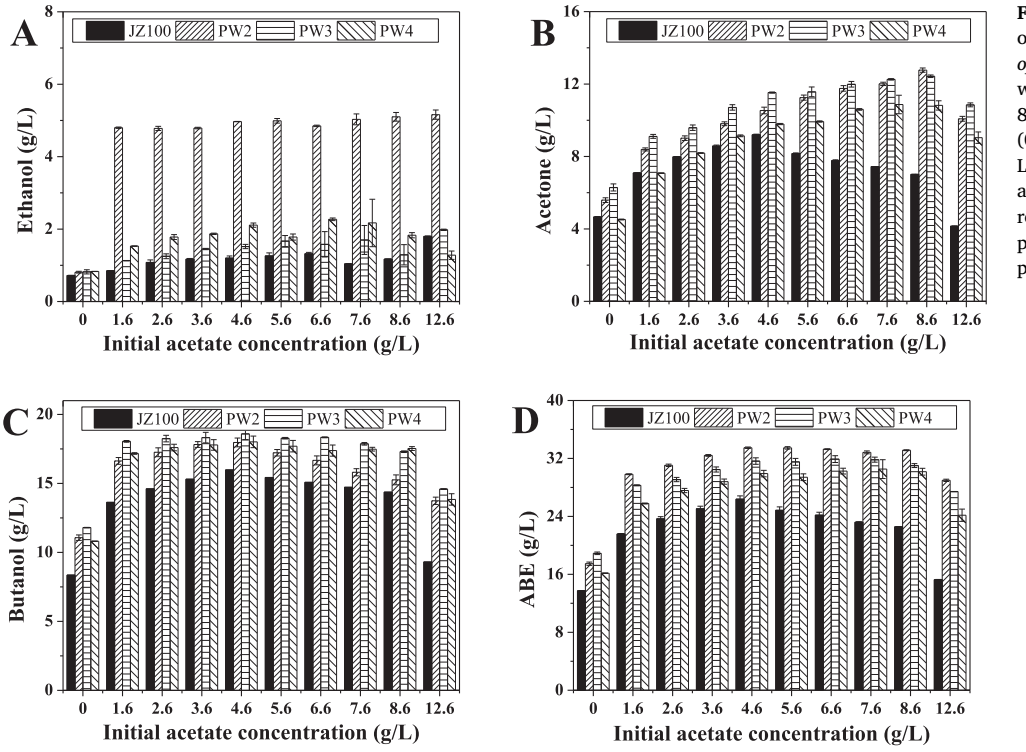

Fig. 2. The effect of supplemented acetate on solvent production by *C. saccharoperbutylacetonicum* mutant strains. Cultures were grown in 100 ml MP2 medium, with 80 g/L glucose and various levels of acetate (0–12.6 g/L acetate; in the form of 0–17.5 g/L sodium acetate). Samples were examined after the fermentation was run for 96 h. The reported value represents the mean of triplicated samples, and the error bar represents the standard deviation.

Table 5
Results of the simultaneous saccharification and fermentation (SSF) with acetic-acid-pretreated switchgrass using various *C. saccharoperbutylacetonicum* mutant strains.*

Results	Strains		
	JZ100	PW2	PW3
Residual sugars (g/L)	3.9 ± 0.1^{a}	3.5 ± 0.1^{ab}	3.3 ± 0.0^{b}
Acetone (g/L)	4.1 ± 0.1^{a}	5.3 ± 0.0^{b}	6.0 ± 0.1^{c}
Ethanol (g/L)	0.7 ± 0.0^{a}	1.8 ± 0.1^{b}	1.2 ± 0.1^{c}
Butanol (g/L)	8.0 ± 0.1^{a}	8.3 ± 0.1^{a}	8.3 ± 0.1^{a}
Final solvents (g/L)	12.8 ± 0.2^{a}	15.4 ± 0.1^{b}	15.4 ± 0.2^{b}
Final solvent yield (g/g)	0.26 ± 0.01^{a}	0.31 ± 0.00^{b}	0.31 ± 0.01^{b}
Acetic acid (g/L)	4.4 ± 0.1^{a}	3.2 ± 0.1^{b}	3.9 ± 0.0^{a}
Butyric acid (g/L)	1.5 ± 0.1^{a}	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}

* All results shown are the average value ± standard deviations from replicated experiments. Values with the same letter within the same row are not significantly different at the 0.05 level based on Tukey's HSD test.

Acknowledgements

This work was supported by the USDA-NIFA Southeastern Sun Grant, the USDA-NIFA Hatch project (ALA014-1017025), and the Alabama Agricultural Experiment Station. We thank Dr. Hans Blaschek (University of Illinois at Urbana-Champaign) for providing pTJ1. We also thank Ceres, Inc. (Thousand Oaks, CA) for providing the Alamo-I switchgrass, and Novozymes North America, Inc. for providing the Cellic CTec2 enzyme.

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