Appl Microbiol Biotechnol
DOI 10.1007/s00253-017-8093-z

BIOENERGY AND BIOFUELS

ARTP mutation and genome shuffling of ABE fermentation symbiotic system for improvement of butanol production

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Received: 18 July 2016 / Revised: 20 December 2016 / Accepted: 23 December 2016
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Abstract Butanol is an ideal renewable biofuel which possesses superior fuel properties. Previously, butanol-producing symbiotic system TSH06 was isolated in our lab, with microoxygen tolerance ability. To boost butanol yield for large-scale industrial production, TSH06 was used as parental strain and subjected to atmospheric and room temperature plasma (ARTP) and four rounds of genome shuffling (GS). ARTP mutant and GS strain were co-cultured with facultative anaerobic *Bacillus cereus* TSH2 to form a symbiotic system with microoxygen tolerance, which was then subjected to fermentation. Relative messenger RNA (mRNA) level of key enzyme gene was measured by real-time PCR. The highest butanol titer of TS4-30 reached 15.63 g/L, which was 34% higher than TSH06 (12.19 g/L). Compared with parental strain, mRNA of acid-forming gene in TS4-30 decreased in acidogenesis phase, while solvent-forming gene increased in solventogenesis phase. This gene expression pattern was consistent with high butanol yield and low acid level in TS4-30. In summary, symbiotic system TS4-30 was obtained with butanol titer improvement and microoxygen tolerance.

Keywords Butanol fermentation · Symbiotic system · TSH06 · TS4-30 · Genome shuffling · Real-time RT-PCR

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Introduction

With the global energy crisis and environmental degradation, growing attention has been devoted to renewable biofuels. Butanol is superior than ethanol owing a higher calorific value, lower corrosivity, and better compatibility with gasoline, which make it an ideal renewable biofuel (Ezeji et al. 2003; Peng 2005; Shen and Ren 2005) as it is directly available to the engine without modification. However, production of biobutanol using microorganism has by far not reached the level of large-scale industrial production yet because of low butanol titer, yield, and productivity (Pereira et al. 2015). Searching for a high butanol-yielding strain is one of the approaches to promote mass production of biobutanol.

To improve butanol production, strains can be modified either by metabolic engineering (Green et al. 1996; Nair et al. 1999; Harris et al. 2000; Tomas et al. 2003; Jiang et al. 2009) or by induced mutagenesis (Zhang et al. 1996; Annous and Blaschek 1991). Metabolic engineering is a direct and

Published online: 10 January 2017

feasible solution to obtain promising trait by targeting specific gene. However, this strategy is based on deep understanding of genetic background of target strains. It is time-consuming and labor-intensive to fully understand the genetic regularity of butanol-producing strain. Butanol fermentation is a complicated process with two distinct phases. Systemic analysis suggested that alteration expression of single solvent-associated gene was insufficient to influence the whole metabolic flux and to enhance butanol production (Haus et al. 2011). In fact, it is effective to target regulation factor (Nair 1998; Harris et al. 2000; Tomas et al. 2003) than key enzyme genes (Green et al. 1996; Jiang et al. 2009) for enhancement of butanol production. This situation is also consistent with the concept of global transcription machinery engineering (gTME) (Alper et al. 2006). Due to complexity of metabolism, it is difficult to obtain a substantial phenotype enhancement by changing a single gene. On the other hand, transcription factors and DNA-binding motifs regulate expression of series of genes, which makes it advantageous to explore the genome through perturbations of this global transcription machinery (Alper and Stephanopoulos 2007). Unfortunately, only a few regulation factors have been studied in butanol-producing strains. Mutagenesis technologies have been successfully used to boost butanol production (Zhang et al. 2002; Gong et al. 2009; Lee et al. 2009). Butanol titer was improved in screening mutant strain compared with original strain (Annous and Blaschek 1991). However, mutant by mutagenesis is random, predominately neutral, or detrimental (Li et al. 2012). Screening of improved strain is laborious after mutagenesis. At the same time, mutants are heterogeneous and harbor different genotype. So, combination of different superior advantage in one strain will be ideal for strain improvement. Genome shuffling (GS) is such a solution with combination of entire genome reorganization and conventional breeding process. This technology use the advantage of parents with genetic diversity, also making multiple excellent characters of offspring appear more often. So, it is an effective method to obtain strains with optimal phenotype (Zhang et al. 2002). Compared with genetic engineering technology, mutagenesis and GS technology do not need to know the genetic background of microbe and also can achieve purpose of rapid breeding. This method has been widely applied to improve production in various microorganisms (Gong et al. 2009; Lee et al. 2009, Otte et al. 2009; Li et al. 2012; Mao et al. 2010; Gérando et al. 2016; Gao et al. 2012; Zhang et al. 2015).

Previously, we have isolated a butanol-producing symbiotic system TSH06. TSH06 consists of two strains: an obligate anaerobic *Clostridium acetobutylicum* TSH1 and a facultative anaerobic *Bacillus cereus* TSH2 (Wang et al. 2015). The symbiotic system is different from the reported strains in that it can produce butanol non-anaerobically. It has extensive adaptability and good fermentation performance. To increase butanol yield, TSH06 was used as parental strain and subjected to atmosphere and room temperature plasma (ARTP) firstly to generate mutant library. ARTP is an effective, safe, and environment benign microbial mutagenesis tool for creating mutant library of microorganisms (Otte et al. 2009; Fang et al. 2013; Li et al. 2014). GS was carried out with strains screening from ARTP. To keep microoxygen tolerance, *B. cereus* TSH2 was added to the mutant and GS strain to form new symbiotic system.

Materials and methods

Microorganisms and media

Symbiotic system TSH06 was isolated and stocked in glycerol at −80 °C in our lab. When used, it was recovered in a corn mash medium (containing 6% corn powder) until bubble rose up at 37 °C in normal incubator. This condition was applied in this study unless stated otherwise. The broth was then inoculated in P2 medium and used as seeds. P2 medium contained (per liter) 80 g/L glucose, 1 g/L yeast extract (autoclave at 115 °C), and 1% each of two nutrient solution: solution 1 $MgSO_4$ 20 g/L, $MnSO_4$ 1 g/L, $FeSO_4$ 1 g/L, NaCl 1 g/L, aminobenzoic acid 0.1 g/L, thiamine 0.1 g/L, biotin 0.001 g/L; solution 2: KH_2PO_4 50 g/L, K_2HPO_4 50 g/L, CH_3COONH_4 220 g/L. Solutions 1 and 2 were sterilization by filter (0.22 μm, Millipore) and added to broth before fermentation.

Atmospheric and room temperature plasma

TSH06 seeds were inoculated in P2 medium and cultured at 37 °C for about 24 h until OD600 approaches 2. Culture liquid (1.5 mL) was centrifuged and resuspended in sterile normal saline solution. For ARTP mutagenesis system, 10 μL diluted culture liquid was transferred to the middle of the sterile metal slide. ARTP plasma mutagenesis was induced under proper voltage. Cells were washed with fresh P2 broth and recovered in P2 medium for 4 h. The culture was then plated on TYA (Used for anaerobic bacteria culture) solid medium (yeast extract 2.0 g/L, beef paste 2.0 g/L, peptone 6.0 g/L, glucose 40 g/L, ammonium acetate 3.0 g/L, KH_2PO_4 0.5 g/L, neutral red 0.2 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $FeSO_4{\cdot}7H_2O$ 0.02 g/L, and agar 15 g/L). TYA plate were incubated in anaerobic chamber (Plas Labs 855AC, USA). Colonies were streaked on a fresh TYA medium supplied with 15 g/L butanol.

Genome shuffling

Mutant cells were cultivated in P2 media until they reached mid-log phase and harvested by centrifugation at 3000×*g* for 10 min. For protoplast formation, 500 μL of the culture was mixed with *Clostridium* protoplasting buffer (CPB), which consisted of RCM (pH 8.5, yeast extract, 3.0 g/L; beef paste,

10.0 g/L; peptone, 10.0 g/L; glucose, 5.0 g/L; soluble starch, 1.0 g/L; sodium chloride, 5.0 g/L; sodium acetate, 3.0 g/L; and cysteine hydrochloride, 0.5 g/L) with 500 mM sucrose, 25 mM $MgCl_2$, 25 mM $CaCl_2$, and 20 mg/L lysozyme. Enzyme treatment was incubated for 1 h at 35 °C. The appearance of spherical cells judged by light microscopy was used as an indicator of protoplast formation. Protoplasts were centrifuged for 5 min at 2000×*g* and washed twice with CPB lacking lysozyme. To generate fusions between protoplast preparations, approximately equal numbers of protoplasts from different strains were mixed, centrifuged, and resuspended in 100 mL CPB lacking lysozyme. Nine volumes of 30 to 60% polyethylene glycol 6000 in CPB were added, and the protoplast mix was incubated for 30 min at room temperature. For regeneration of protoplasts, 5 mL CPB lacking lysozyme was added and the protoplasts were centrifuged, washed, and resuspended in CPB containing 100 mM *N*-acetylglucosamine. Protoplasts were incubated for 5 h at 34 °C and then spread onto regeneration medium TYA. TYA plate were incubated in anaerobic chamber (Plas Labs 855AC, USA) until colonies appeared. Colonies were streaked on fresh TYA supplemented with 20 g/L butanol.

Reconstruction of symbiotic system

Stocked *B. cereus* TSH2 was recovered in LB medium at 37 °C 200 rpm. *B. cereus* TSH2 culture was inoculated together with mutant or GS strains in P2 medium. The co-culture system was incubated statically at 37 °C in normal incubator.

Fermentation

Fermentation was conducted in P2 (40–80 g/L glucose) liquid medium at 37 °C in normal incubator. Each strain was performed as triplicates. A sample was collected every 24 h until the end of fermentation.

Chemical analysis

During the fermentation period, 1.0 mL sample was taken on time and centrifuged at 10,625×*g* for 5 min. The supernatant was used for acetone, butanol, and ethanol (ABE), acetic acid, butyric acid, and glucose concentration analyses. ABE were measured by gas chromatography (GC 2010, Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector (FID) and a glass column (KB-5MS, 25 m × 0.53 mm × 1.00 μm, Kromat Corporation, USA). The temperature of the detector and injector was maintained at 260 and 240 °C, respectively. Each sample was be processed by 10 times dilution and run by 8 min. Isobutanol was added in as internal standard.

Glucose, acetic acid, and butyric acid were determined by high-pressure liquid chromatography (HPLC) analysis (LC-20AT, Shimadzu Co., Kyoto, Japan). A HPX-87 H column (300 × 7.8 mm) (Bio-Rad, USA) was used with a mobile phase of 5 mM sulfuric acid and a flow rate of 0.80 mL/min at 65 °C. Each sample was processed by 100 times dilution and run by 35 min.

Real-time RT-PCR

Real-time PCR of key enzyme genes was performed with 16S ribosomal RNA (rRNA) as interior control for quantification. Primer sequence (Table 1) was designed according to sequence of *C. acetobutylicum* in GenBank. Purity of the RNA was checked using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was reverse-transcripted to complementary DNA (cDNA) as the following system in PCR tube: RNA 2 μL, 5× RT buffer 4 μL, RNase-free water 12 μL, random primer oligo 1 μL. The reaction system was incubated at 60 °C for 5 min. The reaction tube was withdrawn and put on ice for at least 5 min. One microliter RNase inhibitor and 1 μL M-MLV reverse transcriptase (Promega) were added. Reaction was performed at 37 °C for 1 h. cDNA can be subjected to PCR at once or stored at −20 °C until use. Real-time PCR was conducted using Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Richmond, CA). Assay was performed triplicate for each sample. Reaction mixture for each assay contained 12.5 μL of SYBR Premic EX Taq (TaKaRa Biotechnology Co., Ltd), 0.5 μL of each primer, and 2 μL of cDNA in a final volume of 25 μL. PCR was performed using the following protocol: 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, 50 °C for 10 s, and 72 °C for 20 s. When PCR was over, a temperature gradient between 65 and 95 °C was performed for analysis of dissociation curves to verify the specificity of product. The expression level of tested genes was normalized against the expression level of 16S rRNA.

Strain

C. acetobutylicum TSH1 and *B. cereus* TSH2 were deposited in China General Microbiological Culture Collection Center (CGMCC) as CGMCC 8071 and CGMCC 8072, respectively. GS strain *C. acetobutylicum* G4-30 was deposited in China General Microbiological Culture Collection Center (CGMCC) as No. 11771.

Result

Mutant library construction by ARTP

In symbiotic TSH06, *C. acetobutylicum* TSH1 is the only strain able to produce butanol, then the target strain for

Table 1 Primers for real-time PCR

Primer	Sequence
akF	5′-GTATCAAGAACAGCTGCAGA-3′
akR	5′-CTACTGCTGGGTCCATATCT-3′
ptaF	5′-AGAGCCTAAAGTTGCGATGC-3′
ptaR	5′-CCGCTACTTCACTATCTATTGC-3′
bukF	5′-GCGGAGTTGTTAGTTACTTA-3′
bukR	5′-TCTATCCTCTATGGCATTAC-3′
ptbF	5′-TTTCGCACCACCTAAGTTTA-3′
ptbR	5′-GTTACAGATGCAGCGATGA-3′
ctfF	5′-ATACGTGCAGGCGGATCT-3′
ctfR	5′-GTTTCCGGCCTCATCTACAA-3′
thlF	5′-GCACCCTAGATTTGGATCA-3′
thlR	5′-TCAACTCCTGCTGAACCATA-3′
hbdF	5′-TAGTTGCTGATGCCACTTCTG-3′
hbdR	5′-CCGGAACAGTTGACCTTAAT-3′
adhEF	5′-GGGATTAACCGCTTATTCAG-3′
adhER	5′-TACTTATATTGTGGGCAAGG-3′
bdhAF	5′-CCGGTTCAGAGGCAACAC-3′
bdhAR	5′-TGGCATACACCTAAGAAAGC-3′
bdhBF	5′-TGCAGGAGTAGAGCCAAATC-3′
bdhBR	5′-TAACGCTTCTGCCATTCTAT-3′
16SF	5′-GGGCTGCATTTCAAACTGGA-3′
16SR	5′-CGGCACAGAAGGAGTCGATA-3′

mutation. Cells were grown in P2 medium in normal incubator to OD600 ≈ 1. When cell was in exponential growth, most of cells were *C. acetobutylicum* (Wu et al. 2016). ARTP condition was determined by survival rate. Under 120 W, lethality rate surpassed 95%; even radiation time was only 10 s (Fig. 1a). So, voltage was lowered to 100 W. Survival rate was nearly 10% at 100 W for 30 s (Fig. 1b). This condition was chosen for mutation. Cells were subjected to ARTP and then were recovered for 4 h. Mutagenesis cells were spread on TYA solid medium for colony growth. Owing to organic acid forming, colony was bright yellow on TYA medium with neutral red. Bright yellow colonies appeared 3 days later. The yellow colonies were *C. acetobutylicum*, since *B. cereus* TSH2 can hardly grow on TYA medium. Thirty colonies were streaked on a fresh TYA solid medium added with butanol (15 g/L) to improve butanol tolerance, since butanol tolerance is linked to butanol-producing ability. After recovery, 12 colonies grew on a butanol-added medium. The five biggest colonies were picked for fermentation. Other colonies did not grow well with small and atrophic colony on butanol-added media and were not subjected to fermentation. Though mutagenesis cells were tried to cultivate on TYA medium supplied with butanol, no colony grew even after 1 week. This may be attributed to weakness of cell after ARTP treatment. So, cells were recovered on TYA medium firstly; then, colonies were screened on medium supplied with 15 g/L butanol to improve tolerance. Owing to microoxygen tolerance ability, the ARTP procedure was not confined in anaerobic chamber, except for colony growth. Comparing with pure *Clostridium*, most steps can be performed in super clean bench, which was both easy handling and time saving. Colonies growing well on butanol-TYA medium were subjected to fermentation. *B. cereus* TSH2 cells were added to simplify operation. A new symbiotic system was formed with mutant *C. acetobutylicum* and *B. cereus* TSH2. Similar to its parental system, this system has also microoxygen tolerance, which can be operated out of anaerobic chamber. *B. cereus* TSH2 can hardly grow in P2 medium, and it does not consume glucose and influence butanol yield.

Fermentation was performed in Erlenmeyer flask with rubber stopper in normal incubator. Sixty milliliter P2 broth was used in 100-mL Erlenmeyer flask. Fermentation was finished until no bubble rose up and broth became clear. The level of solvents is shown in Fig. 2. Five mutant strains were selected for fermentation. Butanol titer reached 13.42 and 13.67 in mutant A0-1 and A0-2, which were 6.5 and 8.6% higher than the parent system TSH06 (12.60 g/L). Total solvent of mutant A0-3 was higher than that of TSH06, while butanol titer was a litter lower. Both butanol and total solvent of strain A0-4 and A0-5 were less than that of parent TSH06. This result demonstrated that mutagenesis is random, and these mutants were heterogeneous. Improved strains were used then for further genome shuffling to integrate their merit and boost butanol production.

Genome shuffling

For genome shuffling, a genomic library must be obtained which contains various mutations with improvements of the desired phenotype compared with wild type. In this study, butanol yield was the main aim. Three mutants, A0-1, A0-2, and A0-3, were used as starting strains from ARTP mutagenesis with high butanol (15 g/L) tolerance screening and were selected for further genome shuffling. In the first round, the three strains were subjected to shuffling with each other. In total, three combinations were performed. The whole genome of these mutated strains was reorganized randomly by protoplast fusion and further screened for improved strains with target characters for the next round of GS. From the second round of selection, strains were induced to protoplast formation and mixed together for shuffling. During GS, butanol tolerance was still used as a selection marker. After colony appeared on TYA medium, colonies were picked and streaked on TYA medium supplemented with (20 g/L) butanol. Four rounds of GS were conducted in total. At each cycle, about 20 colonies were picked out and assayed for ABE production. Three to four colonies were chosen for the next round of GS according to butanol titer.

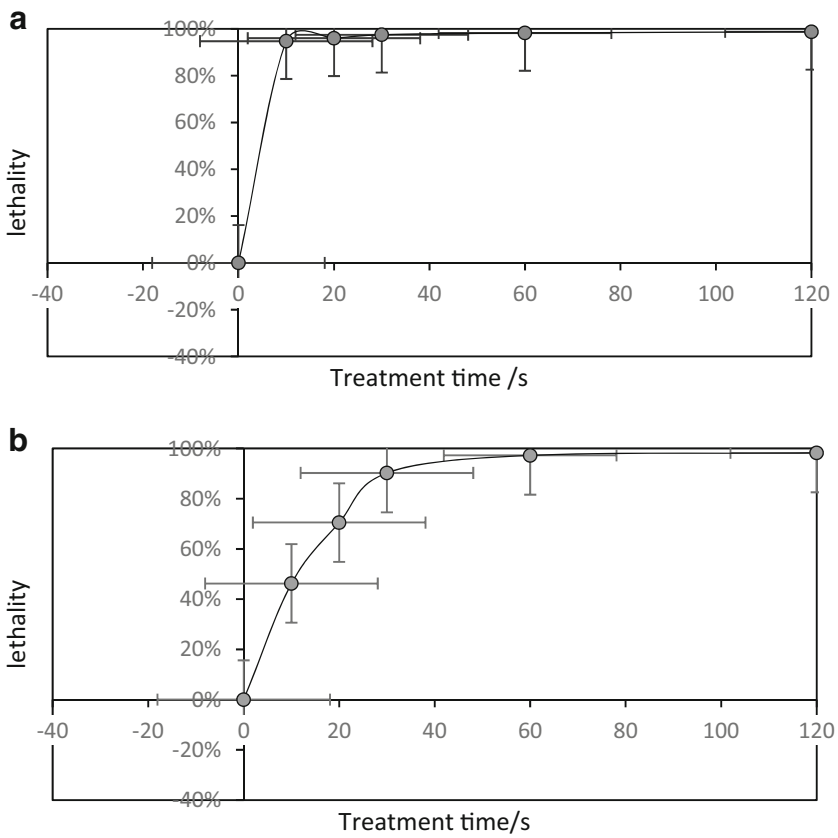

Fig. 1 Lethal rate curve of TSH06 after ARTP treatment. **a** Voltage of 120 W. **b** Voltage of 100 W

Reconstruction of symbiosis with GS strain and *B. cereus* TSH2

In parental symbiotic system TSH06, the role of *B. cereus* TSH2 is to exhaust oxygen in broth and make anaerobic niche for obligate anaerobic, butanol-forming *C. acetobutylicum* TSH1. Butanol yield was not influenced with no butanol-producing ability *Bacillus* (Wang et al. 2015; Abd-Alla and Abdel-Wahab 2012; Trana et al. 2010). *B. cereus* TSH2 does not hamper butanol production in symbiosis TSH06; on the contrary, it accelerates fermentation (Wang et al. 2015). So, when fermentation was carried out with mutant and GS strain, *B. cereus* TSH2 was inoculated in P2 broth to consume oxygen. New symbiotic system was constructed with mutant *C. acetobutylicum* and wild-type *B. cereus* TSH2. *B. cereus* TSH2 was lost during colony recovery and screening in every

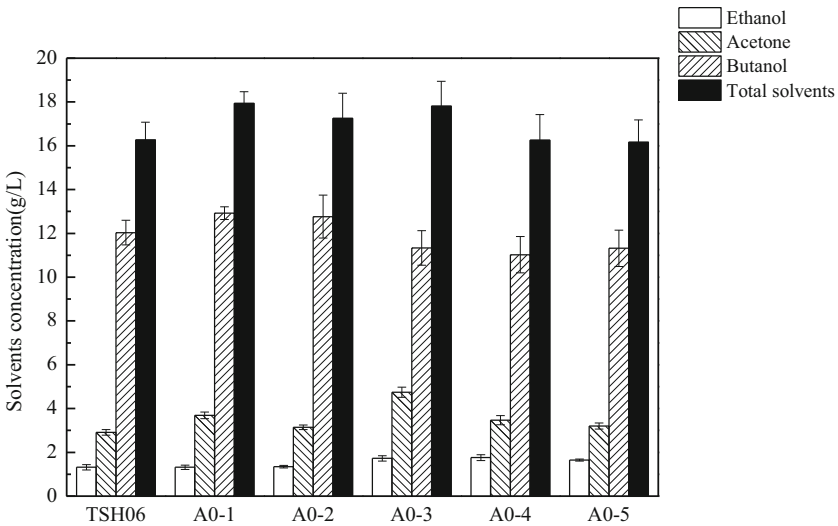

Fig. 2 Fermentation of mutants by ARTP mutagenesis. Fermentation was performed statically with mutant strains (supplied with *B. cereus* TSH2) and TSH06 at 37 °C in normal incubator. Butanol concentration was higher in A0-1 and A0-2 than that in TSH06, while total solvent concentration was higher in A0-3 than that in TSH06. Butanol and solvent concentration were both lower in A0-4 and A0-5 than that in TSH06. A0-1, A0-2, and A0-3 were chosen for genome shuffling

round of GS, when colony was grown on TYA medium in anaerobic chamber. So, it was added again to fermentation broth. The whole procedure is shown in Fig. 3.

Fermentation of GS strain

Fermentation was performed with new symbiotic system with GS strain and *B. cereus* TSH2. GS strain was named as GS*n*-*x*, while symbiotic system with mutant strain named as TS*n*-*x*, where "*n*" denoted the round number of GS and *x* denoted the ordinal number of GS strain. After the fourth round of GS, the superb strain TS4-30 was selected for fermentation in triplets, with TSH06 as control (Fig. 4). Similar trends of glucose consumption and product production capacity were seen in TS4-30 and TSH06. Ultimately, parent strain TSH06 produced 11.96 g/L butanol and 17.3 g/L total solvent with 59.8 g/L glucose consumption, which is comparable to its ordinary level. At the same time, butanol and total ABE reached 15.63 and 20.43 g/L for TS4-30, with 64.8 g/L glucose consumption. Butanol titer of TS4-30 is 30% higher than that of TSH06. Butanol concentration was similar to that of strain screening. This result demonstrated that performance of TS4-30 was significant and stable.

Since 80 g/L original glucose was excessive, fermentation experiments were also conducted in P2 medium with lower glucose concentration and corn meal medium (Table 2 and Fig. 5). In 60 and 40 g/L glucose P2 medium, glucose was almost exhausted at the end of the fermentation. Butanol and ABE concentration was lower relative to the 80 g/L glucose, while butanol yield was similar. In each medium, butanol titer was higher in TS4-30 than that in TSH06. Using a cheaper medium corn meal, TS4-30 produced 10.33 g/L butanol and 15.73 g/L ABE, relative to 9.08 g/L butanol and 12.83 g/L ABE for TSH06. So, TS4-30 demonstrated superiority in different substrate and different glucose concentration.

Transcriptional level of relative genes

As the key enzymes have been studied well for ABE fermentation (Gheshlaghi et al. 2009), it is convenient to measure relative mRNA level of key enzyme gene between GS strain and parent strain. Samples were collected from two time points, 18 and 72 h, representing acidogenesis phase (Fig. 6a) and solventogenesis phase (Fig. 6b), respectively. Real-time PCR was carried out in triplicates for each sample with 16S rRNA as internal control.

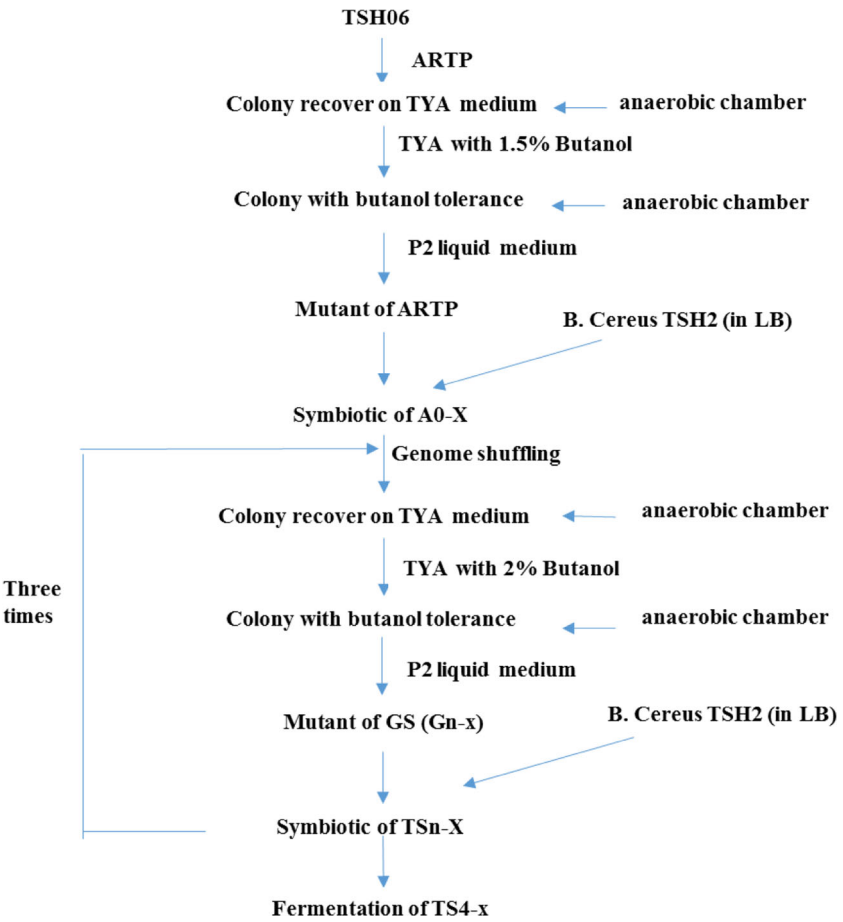

Fig. 3 Reconstruction of symbiotic system with mutant strain and *B. cereus* TSH2. Most of the procedure was conducted out of anaerobic chamber, except that noted. *A0-x* symbiosis of ARTP mutant and *B. cereus* TSH2, *x* ordinal number strain, *GS* genome shuffling, *Gn-x* mutant of genome shuffling, *TSn-x* symbiosis with GS strain Gn-x and *B. cereus* TSH2, *n* = 1–4 round number of GS

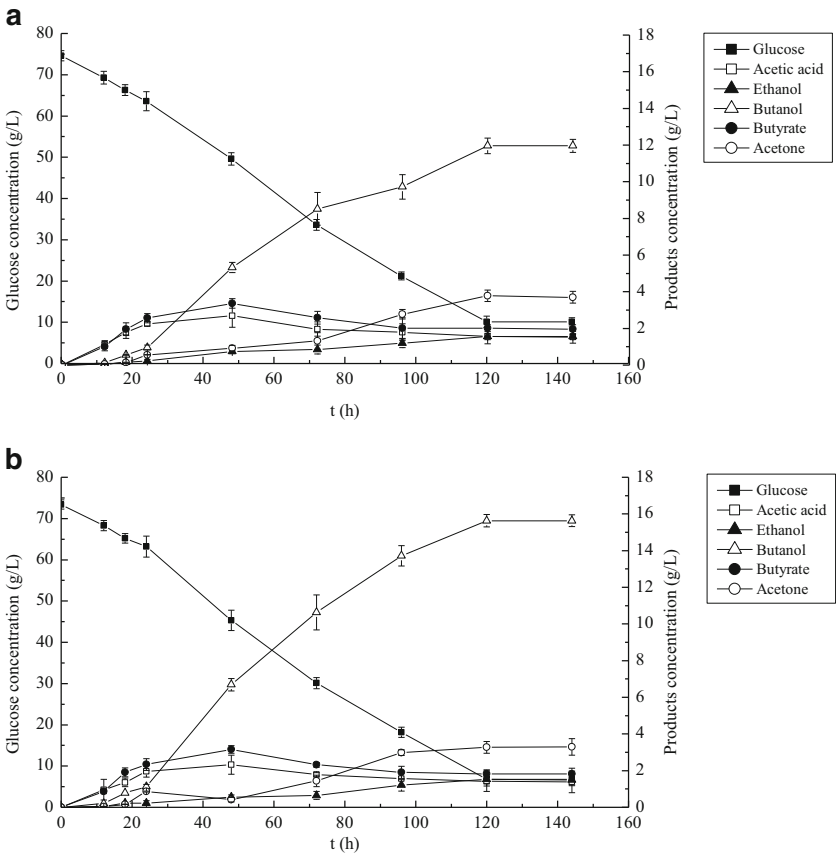

Fig. 4 Comparison of fermentation process of TSH06 and TS4-30. **a** TSH06. **b** TS4-30

Key enzymes were chosen from fermentation metabolic pathways. In acidogenesis phase, phosphate acetyl transferase (*pta*) and acetate kinase (*ack*) are in charge of acetic acid production, while phosphate butyryl transferase gene (*ptb*) and butyrate kinase (*buk*) are in charge of butyrate generation. Enzyme of acid reassimilation is CoA-transferase (*ctf*), which also participates in the formation of acetoacetate from acetoacetyl-CoA, the first and key step of acetone formation. Acetyl-CoA acetyltransferase (*thl*) catalyzes two molecule acetyl-CoA into one molecule acetoacetyl-CoA, a reaction in charge of formation of C4 unit from C2 unit. Acetoacetyl-CoA is the common precursor of acetone, butyrate, and butanol. Hydroxybutyryl-CoA dehydrogenase (*hbd*) belongs to one gene in BCS operon. Protein product of BCS operon catalyzes acetoacetyl-CoA into butyryl-CoA, the common precursor of butyrate and butanol. Three key aldehyde/alcohol dehydrogenase genes (*adhE*, *bdhA*, and *bdhB*) were also detected. In acidogenesis phase (Fig. 6a), key enzyme

Table 2 ABE production and glucose consumption of different media

Glucose	80 g/L (g/L)		60 g/L (g/L)		40 g/L (g/L)		Corn meal (g/L)	
	TS4-30	TSH06	TS4-30	TSH06	TS4-30	TSH06	TS4-30	TSH06
Ethanol	1.53 ± 0.03	1.56 ± 0.04	1.64 ± 0.02	1.03 ± 0.02	1.65 ± 0.02	1.01 ± 0.05	0.72 ± 0.03	0.25 ± 0.01
Acetone	3.27 ± 0.02	3.78 ± 0.02	3.47 ± 0.03	2.12 ± 0.03	2.94 ± 0.04	2.35 ± 0.03	4.69 ± 0.02	3.5 ± 0.05
Butanol	15.63 ± 0.32	11.96 ± 0.81	14.17 ± 0.32	11.02 ± 0.21	9.6 ± 0.12	8.78 ± 0.29	10.33 ± 0.23	9.08 ± 0.25
ABE	20.43 ± 0.36	17.3 ± 1.09	19.29 ± 0.39	14.17 ± 0.63	14.19 ± 0.13	12.14 ± 0.45	15.73 ± 0.24	12.83 ± 0.3
Initial glucose	75.1 ± 1.23	73.9 ± 1.09	55.6 ± 1.23	54.2 ± 1.15	36.2 ± 0.91	35.6 ± 1.02		
Residual glucose	10.3 ± 0.24	14.1 ± 0.32	0.32 ± 0.01	0.22 ± 0.01	0.06 ± 0.01	0.11 ± 0.01		
Glucose consumption	64.8 ± 1.03	59.8 ± 1.03	55.28 ± 1.21	53.98 ± 1.03	36.14 ± 0.86	35.49 ± 1.03		
Butanol yield	0.24 ± 0.01	0.2 ± 0.01	0.26 ± 0.01	0.2 ± 0.01	0.26 ± 0.01	0.25 ± 0.01		

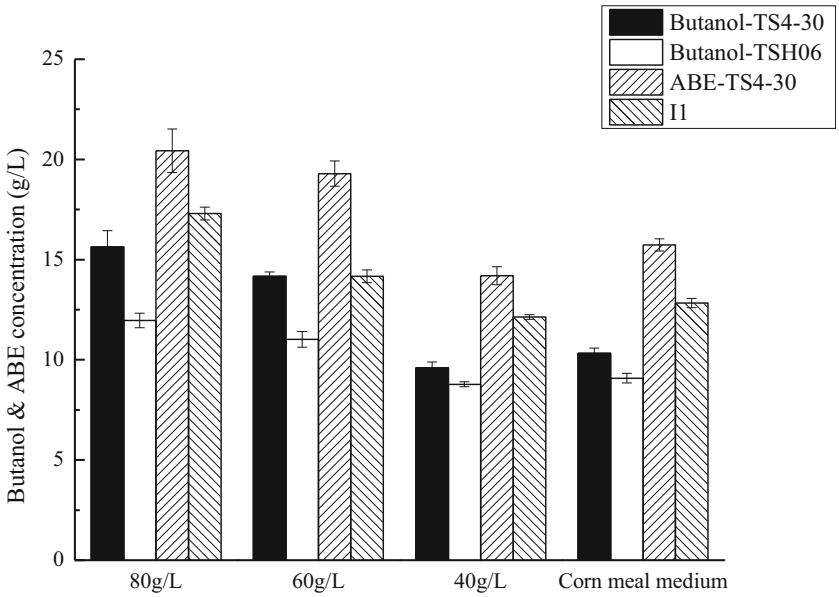

Fig. 5 Comparison of butanol and ABE production in different media

genes for organic acid forming was lower in TS4-30 compared with TSH06. The level of *buk*, *ptb*, *pta*, and *ack* mRNA of TS4-30 was lower than those of TSH06. On the other hand, these genes were higher in solventogenesis phase in TS4-30 (Fig. 6b), though acid was not produced at this stage. It is worth noting that in acid-producing phase, key enzyme gene ctf mRNA level of TS4-30 was quite higher than that of TSH06. This result can partly account for lower organic acid level in GS strain. The level of thl and *hbd* in TS4-30 was lower in acidogenesis phase but higher in butanol-producing phase, compared with those of TSH06. This profile would lead to lower butyl-CoA in acidogenesis phase but higher butyl-CoA solventogenesis phase. Butanol-producing gene *adhE*, *bhdA*, and *bdhB* mRNA levels of TS4-30 were higher both in acidogenesis phase and in butanol-producing phase, compared with those of TSH06. High level of aldehyde/alcohol dehydrogenase gene was consistent with boosted butanol concentration in GS strain. Taken together, in TS4-30, it seemed that acidogenesis genes were inhibited during acidogenesis phase, while acid reflux gene (ctf) was enhanced. In solventogenesis phase, key enzyme gene for solvent forming was all boosted in TS4-30 compared to those in TSH06. Gene expression level was consistent with higher butanol level in TS4-30. However, situation is not always consistent as for acetone and ethanol. Gene *ctf* is responsible for acetone direction. Little change was found in acetone level between TSH06 and TS4-30. Metabolic pathway (Fig. 6c) and key enzyme of ABE fermentation are illustrated in Fig. 6. Gene expression was regulated by transcription factors (TFs). However, little is known about TF in butanol-producing *C. acetobutylicum*. To trace these, TFs will help to understand regulation of butanol fermentation and will ultimately enhance butanol productivity.

Discussion

Butanol toxicity is thought the bottleneck (Lee et al. 2012) which hampers butanol titer in broth and also impedes industrialization of biobutanol (Baer et al. 1987). High butanol tolerance was thought accompanied with high butanol production (Papoutsakis 2008). So, butanol tolerance was applied to screen colonies both in ARTP mutation and GS. Colony cannot grow on medium supplied with butanol directly after ARTP treatment. However, some colonies did grow on butanol supplement medium after it recovered from normal TYA medium. It was postulated that cells were weak after ARTP treatment. Butanol tolerance ability was manifested after cell recovery. In GS step, cells were spread on TYA medium too and then streaked on butanol addition medium to screen high butanol tolerance strain. Enhancing butanol resistance can effectively improve performance of strains in ABE fermentation. In GS, colony can grow on TYA solid medium supplemented with 20 g/L butanol. However, the best butanol titer was only 15.6 g/L, with glucose remained in broth. Similar result was also reported by another study. In GS strain RH8, butanol titer was 15.3 g/L, though it can tolerate 19 g/L butanol (Mao et al. 2010). These results implied that there was still other factors which hindered the improvement of butanol production other than butanol tolerance. In mutagenesis strain BA 101, addition of sodium acetate enhanced butanol production (Chen and Blaschek 1999). Butanol concentration reached 20.9 g/L, which is the highest record in batch fermentation. Glucose-based P2 medium was used in this and Mao's study

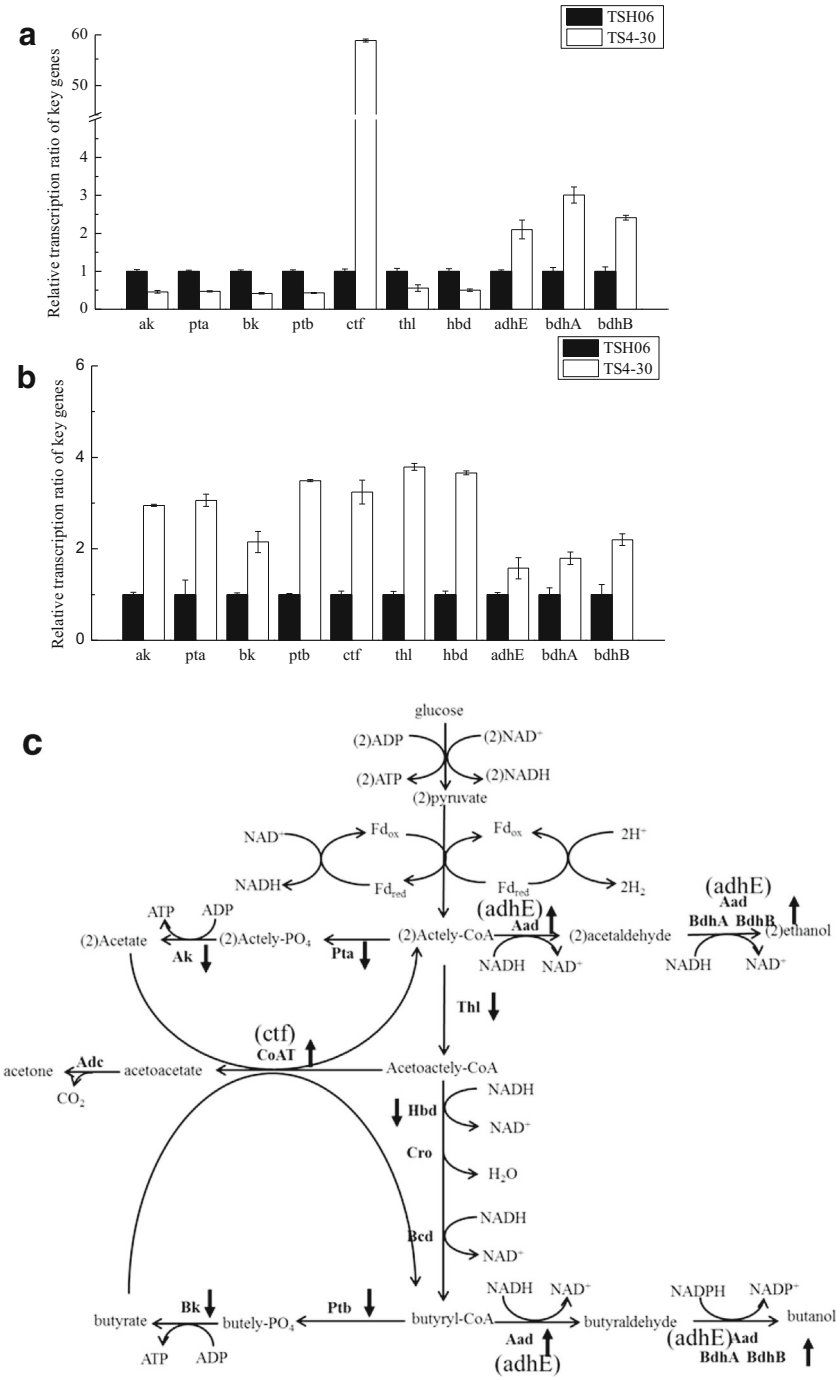

Fig. 6 Relative transcription ratio of key genes at different fermentation phases. **a** Acid-producing phase. **b** Butanol-producing phase. **c** Metabolic pathway of acid-producing phase in the ABE fermentation. *Ak* acetate kinase, *Pta* phosphate acetyltransferase, *Bk* butyrate kinase, *Ptb* phosphate butyryltransferase, *CoAT* CoA transferase (encoded by ctf α and β), *ADC* acetoacetate decarboxylase, *Thl* thiolase, *Hbd* 3-hydroxybutyryl-CoA dehydrogenase, *Cro* crotonase, *Bdc* butyryl-CoA dehydrogenase, *Aad* acetaldehyde-CoA/alcohol dehydrogenase, *BdhA* NADH-dependent butanol dehydrogenase A, *BdhB* NADH-dependent alcohol dehydrogenase B

(Mao et al. 2010), and similar results were obtained. In fact, butanol increased little after the third round of GS. Butanol titer was nearly similar in the third and the fourth round of GS in this study (data not shown). It is unknown if it reaches the limit of butanol production ability for GS strain in normal glucose-based medium. Other factors, such as endospore forming, should be paid take into consideration in the future study (Papoutsakis 2008).

Different with pure *Clostridium*, original strain TSH06 is symbiotic system. Its outstanding merit is non-anaerobic operation, growth, and fermentation. In a study of mutation and GS with *Clostridium beijerinckii*, not only the operation was restricted in anaerobic condition, but also buffer had to been pretreated to anaerobic in advance (Gérando et al. 2016). TSH06 was exempt from strict anaerobic operation, so did reagent used. TSH06 consists of two strains: obligate anaerobic *C. acetobutylicum* TSH1 and facultative anaerobic *B. cereus* TSH2. *C. acetobutylicum* TSH1 is butanol-yielding strain. ARTP mutation and genome shuffling is targeted to *C. acetobutylicum* TSH1 to boost butanol.

TSH06 was used as whole system, because *B. cereus* TSH2 was absolute minority when the cell underwent mutation and shuffling (Wu et al. 2016). At the same time, operation was simplified greatly without anaerobic restriction. Cells were transferred into anaerobic chamber only after they were plated on TYA plate for recovery. *B. cereus* TSH2 was lost both during the process of ARTP and GS, since the colonies were all undergone two rounds of TYA selection under anaerobic condition: one for colony recovery and one for butanol screening. Under anaerobic condition, *B. cereus* TSH2 cannot grow on TYA plate. Under aerobic condition, very small dark colony appeared several days after *B. cereus* TSH2 was streaked on TYA medium. So, the bright yellow colonies were pure *C. acetobutylicum* after screening. It was also verified that mutagenesis strain cannot grow in P2 liquid medium under non-anaerobic condition, while it did grow when *B. cereus* TSH2 was added. This situation was same to parental symbiotic TSH06 (Wang et al. 2015). In this way, new symbiotic system was obtained after ARTP mutation and genome shuffling. Butanol titer was boosted, and oxygen tolerance ability was maintained.

Acknowledgements

Authors' contributions GW, GW, SM, and JZ designed the experiments. CG, GW, and SM performed the experiments. CG, GW, and JZ drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards This article does not contain any studies with human participants performed by any of the authors.

Funding This work was supported by the Foundation of Key Laboratory for Industrial Biocatalysis (Tsinghua University), Ministry of Education (No. 2015302), National Natural Science Foundation of China (No. 21176141), and National Energy Administration (No. 20131448926). We appreciate Prof. Xinhua Xing and his group for their help in ARTP mutation, which is the basis of this article.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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