Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6864-y

BIOENERGY AND BIOFUELS

Isolation and characterisation of non-anaerobic butanol-producing symbiotic system TSH06

Genyu Wang[1] · Pengfei Wu[1] · Ya Liu[1,2] · Shuo Mi[1] · Shuai Mai[1] · Chunkai Gu[1] · Gehua Wang[1] · Hongjuan Liu[1] · Jianan Zhang[1] · Børre Tore Børresen[3] · Evy Mellemsæther[3] · Hans Kristian Kotlar[3]

Received: 30 March 2015 / Revised: 15 July 2015 / Accepted: 18 July 2015

Abstract Butanol-producing microorganisms are all obligate anaerobes. In this study, a unique symbiotic system TSH06 was isolated to be capable of producing butanol under non-anaerobic condition. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S ribosomal RNA (rRNA) revealed that two strains coexist in TSH06. The two strains were identical to *Clostridium acetobutylicum* and *Bacillus cereus*, respectively. They were isolated individually and named as *C. acetobutylicum* TSH1 and *B. cereus* TSH2. *C. acetobutylicum* TSH1 is a butanol-producing, obligate anaerobic strain. Facultative anaerobic *B. cereus* TSH2 did not possess the ability of butanol production; however, it offered *C. acetobutylicum* TSH1 the viability under non-anaerobic condition. Moreover, *B. cereus* TSH2 enhanced butanol yield and speed of fermentation. TSH06 produced 12.97 g/L butanol and 15.39 g/L total solvent under non-anaerobic condition, which is 25 and 24 %, respectively, higher than those of *C. acetobutylicum* TSH1. In addition, TSH06 produced butanol faster under non-anaerobic condition than under anaerobic condition. Butanol accounted for more than 80 % of total solvent, which is higher than the known report. TSH06 was stable during passage. In all, TSH06 is a promising candidate for industrialisation of biobutanol with high yield, high butanol proportion, easy-handling and time-saving system. These results demonstrated the potential advantage of symbiosis. This study also provides a promising strategy for butanol fermentation.

Keywords Butanol · Biofuel · Symbiosis · TSH06 · Non-anaerobic fermentation

Introduction

Energy crisis and environmental deterioration have aroused a research upsurge of renewable biofuel. Biobutanol has shown great promising substitute of gasoline owing to significant properties such as higher energy density, lower corrosivity and mutual solubility with gasoline. Butanol fermentation is also known as acetone-butanol-ethanol (ABE) fermentation because of the main end products of acetone, butanol and ethanol. ABE fermentation by *Clostridia* was once ranked second in terms of large-scale industrial fermentation and was widely conducted during the first half part of the twentieth century (Jones and Woods 1986). *Clostridia* are a group of Gram-positive, spore-forming, obligate anaerobic microorganisms. There are four distinct species of *Clostridia* that have been identified among the industrial production strains: *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum*. All of these strains share similar fermentation metabolism pathways (Lütke-Eversloh and Bahl 2011; Ishizaki et al. 1999). The biochemical pathways for the conversion of carbohydrates to acids and solvents have been well studied and reviewed (Ezeji et al. 2007; Gheshlaghi et al. 2009). ABE fermentation can be divided into two distinctive and successive phases: an acid production phase and a solvent production phase. During acidogenesis phase, *Clostridium*

Genyu Wang and Pengfei Wu contributed equally to this work.

✉ Jianan Zhang
zhangja@tsinghua.edu.cn

Hongjuan Liu
liuhongjuan@mail.tsinghua.edu.cn

[1] Institute of Nuclear and New Energy Technology, Tsinghua University, Beijing 100084, China

[2] Food College of Shihezi University, Shihezi, Xinjiang 832000, China

[3] Statoil Petroleum AS, N-4035 Stavanger, Norway

Published online: 15 August 2015

cells grow exponentially and acetic and butyric acid are produced with ATP formation. In the subsequent solventogenesis, cell growth enters a stationary phase and organic acids produced in acidogenesis phase enter the pathway and are reassimilated to solvent. The major end product of the fermentation is butanol, with acetone and ethanol as minor products. The typical ABE fermentation yields acetone, butanol and ethanol in the ratio of 3:6:1. Due to butanol toxicity, the cells cannot survive with high butanol concentration produced (Baer et al. 1987), which hampers the industrialisation of biobutanol.

One other disadvantage of butanol fermentation is the high oxygen sensitivity of *Clostridia.* Strict anaerobic condition is still being applied 100 years since the host strain was first isolated. Fermentation has to be operated under strict anaerobic environment. Fermentation broth has to be flushed with nitrogen gas before inoculation, and *Clostridium* cells have to be cultured in anaerobic chamber. The situation leads to butanol fermentation both complicated and uneconomic. Oxygen tolerance is one desirable trait for butanol-yielding strains because aerobic metabolism could simplify bioprocessing and enable higher cell densities without accumulating higher acid level (Papoutsakis 2008). Metabolic engineering has been applied to improve the oxygen tolerance of *Clostridium* (Hillmann et al. 2008; Zhu et al. 2011; Dong et al. 2011). Oxygen-tolerance-related gene was chosen to enhance the survival under limited oxygen concentration. Peroxide repressor-homologous protein (PerP) is the repressor protein involved in oxygen defence. Deletion of the *perP* gene prolonged period of aerotolerance in C. acetobutylicum (Hillmann et al. 2008), and also, it is possible to transform *Clostridium* outside of the anaerobic chamber (Dong et al. 2011). Glutathione (GSH) can improve cellular resistance to oxidative stress. Introducing the glutathione synthetase gene *gshAB* from *Escherichia coli* into *C. acetobutylicum* resulted in improved cell survival upon aeration (Zhu et al. 2011). Tolerance of oxygen was improved in these engineering strains to some extent, but not enough to break through the restriction of anaerobic fermentation. Synthetic biology was applied by introducing butanol synthesis pathway into aerobic microorganism. An attempt was made to induce butanol biosynthesis in *Bacillus subtilis* via the introduction of a butanol-synthesis enzyme. The engineering strain produced poor butanol though it harboured all genes for the necessary enzyme to produce butanol (Nielsen et al. 2009). The butanol biosynthesis pathway was also introduced in *E. coli* (Inui et al. 2008) and *Pseudomonas putida* (Nielsen et al. 2009). Butanol could be produced without anaerobic treatment in these strains, but butanol titre was far less than that of natural host strains. These facts indicate that the butanol fermentation metabolism is more complex than expected.

To simplify the operation procedure, non-anaerobic condition was applied to isolated butanol-production microorganism in our lab. By subculture and isolation, butanol-producing microbe was obtained and named as TSH06. TSH06 can effectively ferment starch and glucose to produce ABE. The fermentation process and products were similar to that of *Clostridium* species, except that TSH06 can be cultured under non-anaerobic condition. This is quite different from the conventional butanol-producing *Clostridia.* Fermentation was studied under non-anaerobic and anaerobic conditions. The mechanism of oxygen tolerance was also explored.

Materials and methods

Microbe isolation for butanol production

Samples of soil, corn powder, potato and maize straw were collected from the agricultural areas around the campus and used as source of microorganism. The butanol-producing bacteria isolation procedure was as follows: 1 g of each material was mashed and laced into a sterile test tube. Next, 10 mL of sterile hot (approximately 55 °C) bacteria enrichment reinforced clostridial medium (RCM) solid medium (pH 8.5, containing per litre the following: yeast extract, 3.0 g; beef paste, 10.0 g; peptone, 10.0 g; glucose, 5.0 g; soluble starch, 1.0 g; sodium chloride, 5.0 g; sodium acetate, 3.0 g; cysteine hydrochloride, 0.5 g; and agar, 15 g). The medium was heated to 85 °C and incubated 10 min for heat shock, and the tubes were cooled with running water. The tubes were then placed in a dryer. Gallic acid was placed in the dryer, and 200 mL of 10 % NaOH was poured into the dryer after the tubes were put in. The dryer was sealed and incubated at 37 °C for 120 h. The subculture was conducted by heating the culture medium to 85 °C for 10 min. Then, 0.5 mL of culture was transferred to a freshly sterilised test tube. RCM was poured in and cultured again at 37 °C for 120 h as before. One loop of culture was picked and used to inoculate tryptone-yeast extract-acetate (TYA) solid medium (containing per litre: yeast extract 2.0 g, beef paste 2.0 g, peptone 6.0 g, glucose 40 g, ammonium acetate 3.0 g, KH_2PO_4 0.5 g, neutral red 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.02 g and agar 25 g). The plate was placed in a dryer and incubated at 37 °C until yellow colonies appeared. The colonies were incubated in hemi-synthesis P2 medium (containing per litre: glucose 30–60 g and yeast extract 1 g (solution I); KH_2PO_4 50 g, K_2HPO_4 50 g and ammonium acetate 220 g (solution II); and para-amino-benzoic acid 0.1 g, thiamin 0.1 g, biotin 0.001 g and $MgSO_4 \cdot 7H_2O$ 20 g, $MnSO_4 \cdot H_2O$ 1 g, $FeSO_4 \cdot 7H_2O$ 1 g and NaCl 1 g (solution III; vitamin and mineral solution)). Solutions I and II were autoclaved separately. Solution III was filter sterilised with a 0.22-μm pore size filter (Millipore). Solutions II and III were added into solution I before inoculation, giving a final concentration of 1 % (vol/vol). The bacteria were subcultured in P2. The strains that produced gas quickly with turbid medium

were picked for further study. The screened strains were passaged in P2 medium for eight generations to evaluate the fermentation stability.

DGGE of 16S rRNA and phylogenetic analysis

Genomic DNA was extracted according to the manufacturer's instructions of the used kit (TaKaRa MiniBest Bacterial Genome DNA Extraction Kit ver. 3.0). A high variable range of 16S ribosomal RNA (rRNA) fragment was amplified according to manufacturer's instructions (Bio-Rad PTC200). In a total reaction volume of 50 μL, the following components were added: 5 μL of 10× polymerase chain reaction (PCR) buffer, 1 μL of dNTPs (10 mM), 1 μL of each primer (u-16SF and u-16SR, Table 1), 50 ng of chromosomal DNA template and 1 μL of Taq polymerase, with the remaining volume being H_2O. PCR was performed in a PTC-100 programmable thermal cycler (Bio-Rad) with the following program: 5 min denaturation at 95 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C. A final extension of 7 min at 72 °C was added at the end. Denaturing gradient gel electrophoresis (DGGE) was performed similar to the process of Wongwilaiwalina et al. (2010). In brief, PCR products were loaded on an 8 % (wt/vol) polyacrylamide gel (acrylamide/*N*,*N*′-methylene-bisacrylamide ratio, 37.3:1) in 1× TAE buffer. The denaturing gradient in the gel was formed by mixing two stock solutions of 8 % acrylamide containing 40 % denaturing agent (2.8 M urea (Amresco), 18.7 % formamide deionised (Amresco)) and 60 % denaturing agent (4.2 M urea, 24 % formamide). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60 °C with the DGGE system (Bio-Rad Laboratories) for 10 h. The gel was washed with Milli-Q water after the end of electrophoresis. The separated DNA was stained with Gelred (Biotum Company) for 30 min and visualised using GelDoc XR system (Bio-Rad). The DNA bands in the DGGE gel were cut out with a razor blade, and DNA was eluted overnight in a 1.5-mL Eppendorf tube containing 100 μL of TAE buffer at 4 °C. DNA was amplified by PCR with the primers for DGGE. PCR product was ligated into a pEasy-T1-Cloning vector (Transgen Biotech) and transformed into *E. coli.* The plasmid was sequenced (Life technology) and compared with

Table 1 Oligonucleotide primers, plasmid and strain used in this study

Strain, plasmid and primer	Relevant character	Source
Strain	*E. coli* DH5α	Transgen Biotech
	Symbiotic system TSH06	This study
	C. acetobutylicum TSH1	This study
	B. cereus TSH2	This study
	C. acetobutylicum ATCC 824	ATCC
Plasmid	pEasy-T1	Transgen Biotech
u-16SF	5′-CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGACTCCTACGGGAGGCA-3′	Wongwilaiwalina et al. (2010)
u-16SR	5′-ATTACCGCGGCTGCTGG-3′	
*B*16SF	5′-GTTGAATAAGCTGGCACC-3′	This study
*B*16SR	5′-CGTGGGCTTTCACATCAGA-3′	
*C*16SF	5′-GGGCTGCATTTCAAACTGGA-3′	This study
*C*16SR	5′-CGGCACAGAAGGAGTCGATA-3′	
B.c ptbF	5′-GAGCTGGTCCAAGTGTTGT-3′	This study
B.c ptbR	5′-CCACTTGCTTTAGATAATGC-3′	
B.c akF	5′-TTGTGAAGTGGTGCTAAATC-3′	This study
B.c akR	5′-TGCCAAGTGAAACAGTATTA-3′	
B.c ctfF	5′-GTATACCATCGCTTGTACCA-3′	This study
B.c ctfR	5′-TTCCATTACTGCCATATCC-3′	
B.c thlF	5′-ATGCATACAACTTTAGAAAG-3′	This study
B.c thlR	5′-TTAGTGAACTTCAATCATCA-3′	
C.a adh1F	5′-GGGATTAACCGCTTATTCAG-3′	This study
C.a adh1R	5′-TACTTATATTGTGGGCAAGG-3′	
C.a crtF	5′-CCCATTCCAACTAATCTT-3	This study
C.a crtR	5′-TAAATGCGTTAAATAGTGAT-3′	
C.a bdhBF	5′-TGCAGGAGTAGAGCCAAATC-3′	This study
C.a bdhBR	5′-TAACGCTTCTGCCATTCTAT-3′	
C.a adcF	5′-GCCCTAGAATATGTGAGCTT-3′	This study
C.a adcR	5′-AACTTCAGCTCTAGGCAATA-3′	

the available 16S rRNA sequenced in GenBank using the NCBI Blast program. Bacterial sequences closely related to those sequences were used in the phylogenetic analysis. The relationship analysis between the strain and related species was performed using ClustalW and Mega 4.1.

Isolation and verification of the components in the microflora

To isolate the individual strain from the microflora, the cells were cultured in P2 liquid medium in an anaerobic chamber (10 % H_2, 5 % CO_2, 85 % N_2; Plas labs 855AC, USA) or in RCM liquid medium (RCM solid medium without agar) in a common incubator. In the anaerobic chamber, the cells were streaked on TYA solid medium. A single colony was picked and inoculated in P2. For the aerobic cultures, cells were streaked on RCM solid medium in a super clean bench. A single colony was picked to inoculate RCM liquid. This subculturing was repeated five times. All of the cells were grown at 37 °C.

Species gene-specific PCR was conducted to verify the isolated strain. The reaction mixture contained 0.5 μL of Premix Ex Taq ver. 2.0 (TaKaRa Biotechnology (Dalian) Co., Ltd), 2.5 μL of Taq buffer, 1 μL of each primer and 0.5 μL of genome DNA in a final volume of 25 μL. Touchdown PCR was used to amplify genes. The protocol was as follows: 95 °C for 2 min, followed by total 35 cycles at 95 °C for 10 s, annealing from 55 to 50 °C for 30 s and 72 °C for 30 s. The PCR product was ligated into a pEASY-T1 Cloning vector using a kit (Transgen Biotech) for sequencing (Life technology). The sequence was subjected to Blast. TSH06 genome from RCM, P2 and LB (containing per litre: tryptone 10.0 g, yeast extract 5.0 g and NaCl 2.0 g) liquid media was used as control. All primers were listed in Table 1.

Real-time PCR analysis

Real-time PCR of 16S rRNA was performed. The purity of the DNA was checked using a NanoDrop ND-2000 spectrophotometer (NanoDrop technologies, Wilmington, DE). Real-time PCR was conducted using the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Richmond, CA). The assay was performed in 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 20 ng of genomic DNA 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 and 55 °C for 30 s. After the PCR was over, a temperature gradient between 65 and 95 °C was performed for analysis of dissociation curves. The plasmid containing 16S rRNA was used as standard to calibrate the amplification efficiency.

Fermentation experiment

Solvent production was determined when the cells were grown in P2 medium and 6.5 % corn mash in an Erlenmeyer flask with a rubber stopper; 6.5 % corn mash was autoclaved at 121 °C for 15 min. The P2 medium contained (g/L) 30–60 g initial glucose according to the individual experiment purpose. The Erlenmeyer flask was put in anaerobic chamber and ordinary incubator for anaerobic fermentation and non-anaerobic fermentation, respectively. Batch fermentation was incubated statically at 37 °C. A total of 60 mL of P2 was used in a 100-mL Erlenmeyer flask; 7 % of seed cultures were inoculated. Samples were withdrawn every 24 h. The concentrations of acetone, butanol and ethanol were determined using a gas chromatograph (Shimadzu GC 2010). The concentrations of glucose, acetic acid and butyric acid were determined using a high-pressure liquid chromatograph (HPLC-20A).

Alcohol tolerance assays

Butanol tolerance was detected using colony forming unit (CFU) assays according to the reported procedure (Zingaro and Papoutsakis 2013). Briefly, cultures were started from a single colony in 5 mL medium. The seeds were used to inoculate a preculture. Preculture was prepared either with 3 % ethanol in order to precondition the cells or without any alcohol supplement. Cultures were serially diluted in tenfold steps in appropriate media. Dilution level was determined based on OD_{600} measurements of the samples. Fifty microlitres of the final dilution was then plated on solid medium plus solvent stressor butanol (1, 1.25 g/L). CFUs were measured after colony appearance.

GenBank accession numbers

16S rRNA of strains isolated in this study was submitted to GenBank. GenBank accession numbers were KR080514 for *C. acetobutylicum* TSH1 and KR080515 for *Bacillus cereus* TSH2, respectively.

Results

Isolation of butanol-producing, non-anaerobic microbe TSH06

Isolation of microorganisms for butanol production was performed at non-strict anaerobic condition from four different materials: soil, corn powder, potato and maize straw. The strains were screened by repeated inoculation between RCM liquid and TYA solid medium. Colonies of bright yellow were selected for fermentation in corn mash medium. Corn mash

was digested and bubble rose up from the bottom. Strains of SJ, CS1, P031 and TSH06 were chosen from each of the four sources. Butanol accounted for more than 60 % of the end product in all these strains (Table 2). The best performance was TSH06, a strain isolated from corn powder; 8.96 g/L butanol was obtained and butanol ratio reached 72.6 %. So, TSH06 was chosen for further study.

During the whole process of isolation, all the operation was kept away from strict anaerobic condition. Cell growth and fermentation was also conducted statically in an Erlenmeyer flask with a rubber stopper in a dryer with NaOH and gallic acid. Compared with the traditional butanol-producing strains, the prominent characteristic of TSH06 is that it can produce butanol from initially non-strict anaerobic conditions. This simplified the procedure. The obtained strains seemed not to relay on the anaerobic condition after they were isolated. All the operation can be conducted in super clean bench, and fermentation can be performed in normal incubator. This simplifies butanol fermentation procedure.

To test if the non-anaerobic character can be kept during passage, TSH06 was subcultured in P2 medium under non-anaerobic conditions. The butanol, total solvent concentration and solvent ratio were kept similar after eight generations. This result demonstrated that the butanol-producing and non-anaerobic characters were both "inheritable." TSH06 was stable for butanol production under non-anaerobic conditions.

DGGE analysis of butanol-producing TSH06

Though the known strains for ABE fermentation are all anaerobic *Clostridium*, the ABE-producing TSH06 can grow under aerobic conditions. One following question is if TSH06 was some new type of microbe which is different from the *Clostridium*. So, DGGE of 16S rRNA was conducted with TSH06 genome from different medium. Two distinct bands appeared on the gel without any unspecific bands (Fig. 1). The bands were cut from the gel separately and subjected to PCR. PCR product was ligated into T-vector. Five colonies were subjected to sequence for each band. Sequences of one band were all identical to *C. acetobutylicum*. To further confirm this strain, species-specific gene primers of *adc*, *ptb*, *ak* and *adhE* in *C. acetobutylicum* were used to verify this strain by PCR (Fig. 2a–d). Sequences of these genes were identical to the known sequence of *C. acetobutylicum* ATCC 824, DSM 1731 and EA 2018. This strain was designated as *C. acetobutylicum* TSH1. Another band was analogous to three species of *Bacilli*: *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis*. To confirm the identity of the *Bacillus*, specific primers of gene *ptb*, *ak*, *ctf* and *thl* were subjected to PCR (Fig. 2e–h), sequenced, and Blast. Sequence results were identical to *B. cereus*. The *Bacillus* strain was designated as *B. cereus* TSH2. This result demonstrated clearly that TSH06 was not a single strain, though it was isolated from one single colony. In fact, it consisted of two strains: *C. acetobutylicum* and *B. cereus*. Phylogenetic tree analysis was shown in Fig. 3. Members of *Clostridium* are obligate anaerobes, whereas members of *Bacillus* are facultative anaerobes. There are four types of butanol-forming *Clostridium*. The other three species *C. beijerinckii*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* are closer when compared with *C. acetobutylicum*. *C. acetobutylicum* TSH1 was analogous to *C. acetobutylicum* according to 16S rRNA sequence. 16S rRNA sequence had been submitted to GenBank. The accession numbers were KR080514 and KR080515 for *C. acetobutylicum* TSH1 and *B. cereus* TSH2, respectively.

Isolation of the two strains in TSH06

The two components of TSH06, *C. acetobutylicum* TSH1 and *B. cereus* TSH2, were obligate and facultative anaerobic microbe, respectively. So, isolation of the two strains was performed anaerobically for *C. acetobutylicum* TSH1 and aerobically for *B. cereus* TSH2 separately. Isolation was repeated for five generations. The last strain was chosen for verification. *B. cereus*-specific *ptb* gene and *C. acetobutylicum*-specific *adc* gene were used for detection of the isolated strain. PCR results were shown in Fig. 4. Genome of TSH06 was also extracted from RCM, LB and P2 liquid medium and used as control. In the isolated *C. acetobutylicum* TSH1 genome, *C. acetobutylicum*-specific *adc* gene could be amplified, whereas *B. cereus*-specific *ptb* gene could not. On the contrary, *B. cereus*-specific *ptb* could be amplified from the isolated *B. cereus* TSH2 genome, but *C. acetobutylicum*-specific *adc* gene could not. The two genes could be amplified from TSH06 genome in all three media of RCM, LB and P2. These results demonstrate that butanol-producing TSH06 contains two strains. Isolated *C. acetobutylicum* TSH1 was pure *C. acetobutylicum* species, and isolated *B. cereus* TSH2 was pure *B. cereus* species. Thus, the two strains of *C.*

Table 2 Solvent production of isolated strains in corn mash medium

Strain	Source	Butanol (g/L)	Total solvent (g/L)	Butanol ratio (%)
SJ	Soil	7.95	12.72	62.7
CS1	Wheat straw	5.61	8.58	65.4
P031	Potato	6.06	9.03	67.1
TSH06	Corn powder	8.96	12.35	72.6

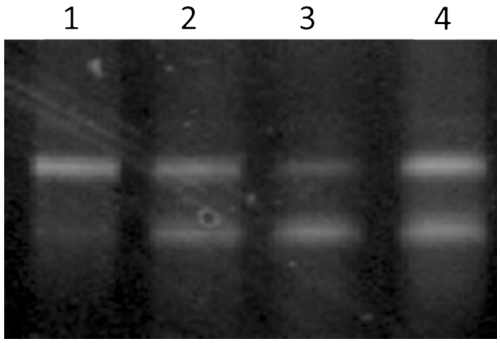

Fig. 1 16S rRNA DGGE analysis of TSH06. There are two bands on the DGGE gel. *1*, DNA from LB culture; *2*, DNA from RCM culture; *3*, DNA from corn mash; *4*, DNA from P2 culture

acetobutylicum TSH1 and *B. cereus* TSH2 were isolated respectively from TSH06. *C. acetobutylicum* TSH1 and *B. cereus* TSH2 were deposited in China General Microbiological Culture Collection Center (CGMCC) as CGMCC 8071 and CGMCC 8072, respectively.

Growth and fermentation of *C. acetobutylicum* TSH1, *B. cereus* TSH2 and TSH06

To reveal which strain is in charge of butanol-production, *C. acetobutylicum* TSH1, *B. cereus* TSH2 and the original symbiotic TSH06 were cultured in different media. Under anaerobic conditions, *C. acetobutylicum* TSH1 alone can grow in corn and P2 medium to produce ABE. *C. acetobutylicum* TSH1 can grow in RCM liquid medium without fermentation (Table 3). Under aerobic conditions, *C. acetobutylicum* TSH1 individually cannot grow in any medium. *B. cereus* TSH2 can grow in RCM medium both aerobically and anaerobically. However, *B. cereus* TSH2 cannot grow in corn or P2 medium under either condition. TSH06, containing the two strains, can grow in all of these three media both non-anaerobically and anaerobically. In addition, TSH06 can ferment corn and glucose both non-anaerobically and anaerobically. These results demonstrate that *C. acetobutylicum*

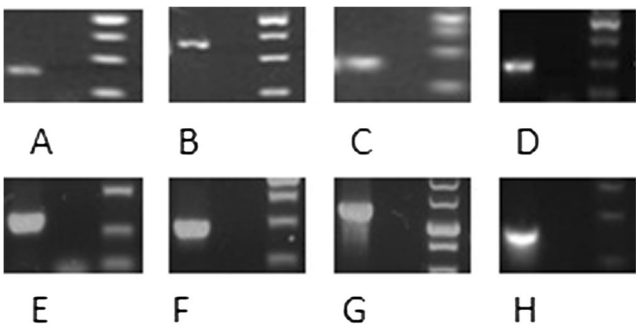

Fig. 2 PCR with species-specific gene primers of *C. acetobutylicum* and *B. cereus*. **a** Gene *adh1* of *C. acetobutylicum*; **b** gene *bdhB* of *C. acetobutylicum*; **c** gene *crt* of *C. acetobutylicum*; **d** gene *adc* of *C. acetobutylicum*; **e** gene *ak* of *B. cereus*; **f** gene *ctf* of *B. cereus*; **g** gene *thl* of *B. cereus*; **h** gene *ptb* of *B. cereus*

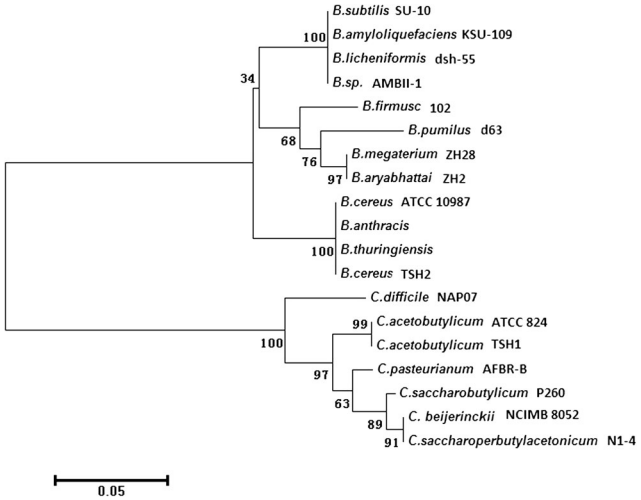

Fig. 3 Phylogenetic tree of the isolated strains and 17 close relatives of known species of *Clostridium* and *Bacillus*. Analysis was based on 200 bp in the 16S rRNA sequence. The tree was constructed using a neighboring joint algorithm. The *scale bar* represents 0.05 substitutions per nucleotide position. *Numbers at the nodes* are the bootstrap values (%)

TSH1 was the strain responsible for the ABE fermentation, while it is obligate anaerobe which cannot grow aerobically. *B. cereus* TSH2 is facultative anaerobic microorganism, which can hardly grow in the glucose-based medium, let alone fermentation. TSH06, co-culture of the two strains, can produce butanol from glucose or starch both non-anaerobically and anaerobically.

Relationship of *Bacillus* and *Clostridium* in the butanol-producing system

Fermentation experiments were conducted to investigate the interaction between *C. acetobutylicum* TSH1 and *B. cereus* TSH2 in the butanol-producing system. *C. acetobutylicum* TSH1 was grown in P2 anaerobically. The cells were then inoculated in P2 and grown aerobically. *B. cereus* TSH2 was grown in LB liquid medium aerobically at 37 °C, 200 rpm, and overnight. Culture of *B. cereus* TSH2 was treated as follows: *B. cereus* TSH2 cells were cultured in LB medium. Cells were collected by centrifugation. Cell-free filtrate was

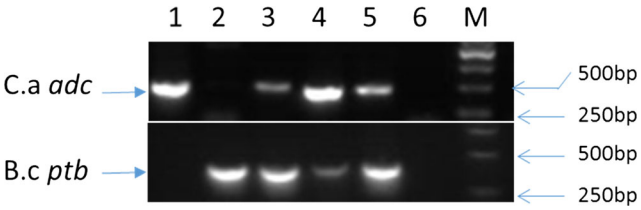

Fig. 4 Species-specific gene PCR with genome of *C. acetobutylicum* TSH1, *B. cereus* TSH2 and TSH06 *C.a adc*, *C. acetobutylicum adc* gene. *B.c ptb*, *B. cereus ptb* gene. *1*, *C. acetobutylicum* TSH1 genome; *2*, *B. cereus* TSH2 genome; *3*, TSH06 genome from RCM medium; *4*, TSH06 genome from P2 medium; *5*, TSH06 genome from LB medium; *6*, water control; *M*, marker

Table 3 Growth and fermentation of TSH06, *C. acetobutylicum* TSH1 and *B. cereus* TSH2 in P2, RCM and corn mash liquid medium

	Medium	TSH06		*C. acetobutylicum* TSH1		*B. cereus* TSH2	
		Growth	Fermentation	Growth	Fermentation	Growth	Fermentation
Anaerobic	P2	+	+	+	+	−	−
	RCM	+	−	+	−	+	−
	Corn mash	+	−	+	+	−	−
Non-anaerobic	P2	+	+	−	−	−	−
	RCM	+	−	−	−	+	−
	Corn mash	+	+	−	−	−	−

gathered from the *B. cereus* TSH2 culture, the crude enzyme extract of *B. cereus* TSH2 was obtained via ultrasonication and *B. cereus* TSH2 culture was autoclaved. All of the above treatments of *B. cereus* TSH2 were added to *C. acetobutylicum* TSH1 in P2 medium separately and kept statically in a conventional incubator chamber at 37 °C. *C. acetobutylicum* TSH1 was cultured alone as a negative control. *C. acetobutylicum* TSH1 could be activated successfully after *B. cereus* TSH2 cells were added, both in the form of the cell precipitate and whole cell culture (Table 4). On the contrary, *C. acetobutylicum* TSH1 could not be rescued by cell-free filtrate, crude enzyme extract, sterilised *B. cereus* TSH2 cell culture or LB medium. *C. acetobutylicum* TSH1 could not grow aerobically alone either. The results demonstrate that it was the *B. cereus* TSH2 cells that offer *C. acetobutylicum* TSH1 the viability under non-strict anaerobic condition, but not the components of the cells.

Fermentation of TSH06 and *C. acetobutylicum* TSH1

Symbiotic TSH06 contained two strains, butanol-producing *C. acetobutylicum* TSH1 and no butanol-producing *B. cereus* TSH2. One will wonder if *B. cereus* TSH2 will influence butanol yield. So, batch fermentation was conducted with the isolated *C. acetobutylicum* TSH1 and TSH06 in P2 medium. Fermentation of *C. acetobutylicum* TSH1 was performed in anaerobic chamber. Fermentation of TSH06 was conducted in anaerobic chamber and in normal incubator at the same time. All the fermentation was performed in a 100-mL Erlenmeyer flask with 60 mL P2 medium (60 g/L original glucose, and 10 % or so was lost after autoclaving). The flask was covered with rubber stopper and kept statically at 37 °C. Each experimental condition was carried out in triplicate. For anaerobic fermentation, samples were collected in anaerobic chamber. For non-anaerobic fermentation, samples were withdrawn in super clean bench. All the fermentation results were illustrated in Fig. 4. *C. acetobutylicum* TSH1 produced 10.39 g/L butanol (Fig. 5a). TSH06 produced 12.38 g/L (Fig. 5b) and 12.97 g/L (Fig. 5c) butanol under anaerobic and non-anaerobic conditions, respectively. The results demonstrated clearly that TSH06 produces more butanol than *C. acetobutylicum* TSH1. The presence of *B. cereus* TSH2 did not decrease butanol production. Furthermore, it promoted butanol production. In all fermentation, acetone and ethanol concentrations were less than 2 and 1 g/L, respectively, in the final broth, which resulted in high butanol proportion. Butanol ratio reached 83, 85 and 84 % for *C. acetobutylicum* TSH1, TSH06 under anaerobic condition and TSH06 under non-anaerobic condition, respectively. For TSH06, butanol tier was slightly higher under non-anaerobic condition than under anaerobic condition. The result was amazing when comparing the fermentation speed. In anaerobic chamber, fermentation was finished in 144 h both for *C. acetobutylicum* TSH1 and TSH06. For TSH06, 12.97 g/L butanol was produced under non-anaerobic condition at 120 h, while only 8.49 g/L butanol was produced under anaerobic condition at the same time. Under anaerobic condition, TSH06 produced 12.38 g/L butanol until 144 h. In fact, butanol concentration had reached 12.58 g/L early at 96 h under non-anaerobic condition. So, fermentation of

Table 4 Rescue function of addition of *B. cereus* TSH2 ingredient on *C. acetobutylicum* TSH1

Addition ingredient	Growth	Fermentation
LB medium	No	No
Cell-free filter of *B. cereus* TSH2	No	No
Autoclaved *B. cereus* TSH2	No	No
Crude enzyme extract by supersonic treatment	No	No
Sediment of *B. cereus* TSH2 cell	Growth well	Fermentation
Whole culture of *B. cereus* TSH2 in LB liquid medium	Growth well	Fermentation

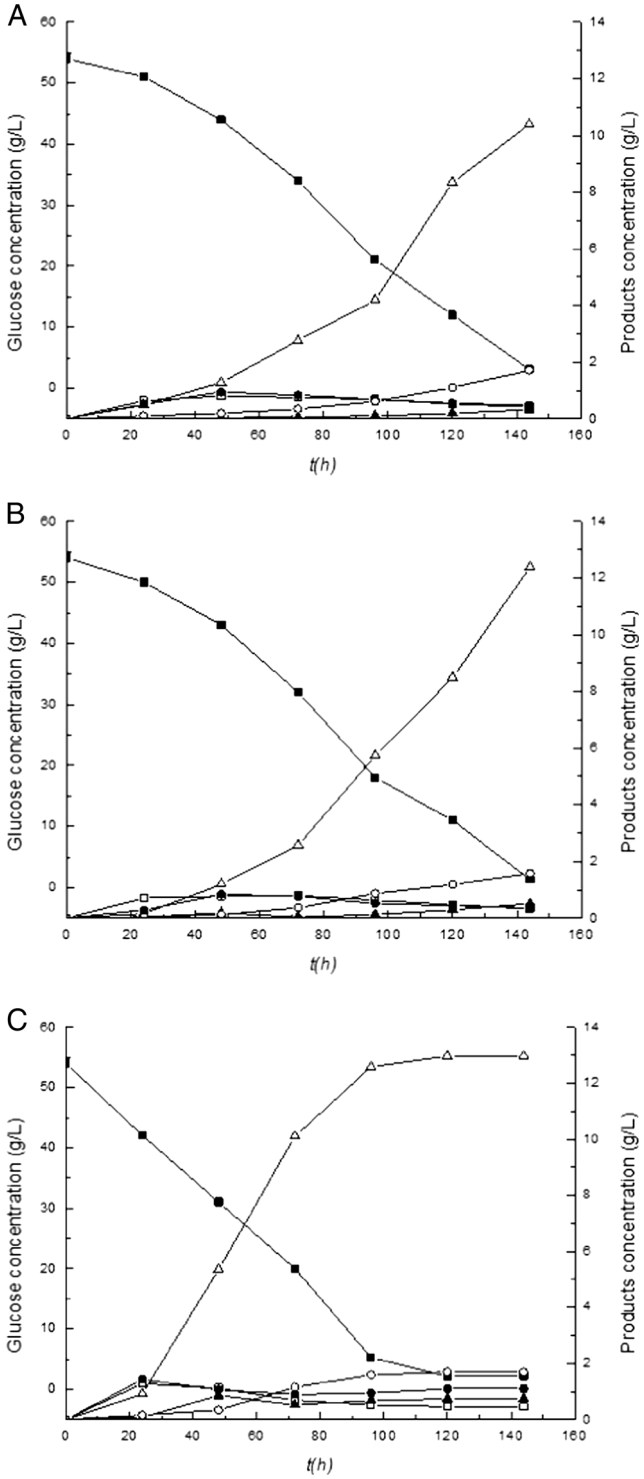

Fig. 5 Fermentation of *C. acetobutylicum* TSH1 and TSH06 in P2 medium. **a** Anaerobic fermentation of *C. acetobutylicum* TSH1; **b** anaerobic fermentation of TSH06; **c** non-anaerobic fermentation of TSH06. *Open rectangle*, glucose; *solid rectangle*, acetic acid; *open circle*, butyric acid; *solid circle*, acetone; *solid triangle*, butanol; *open triangle*, ethanol

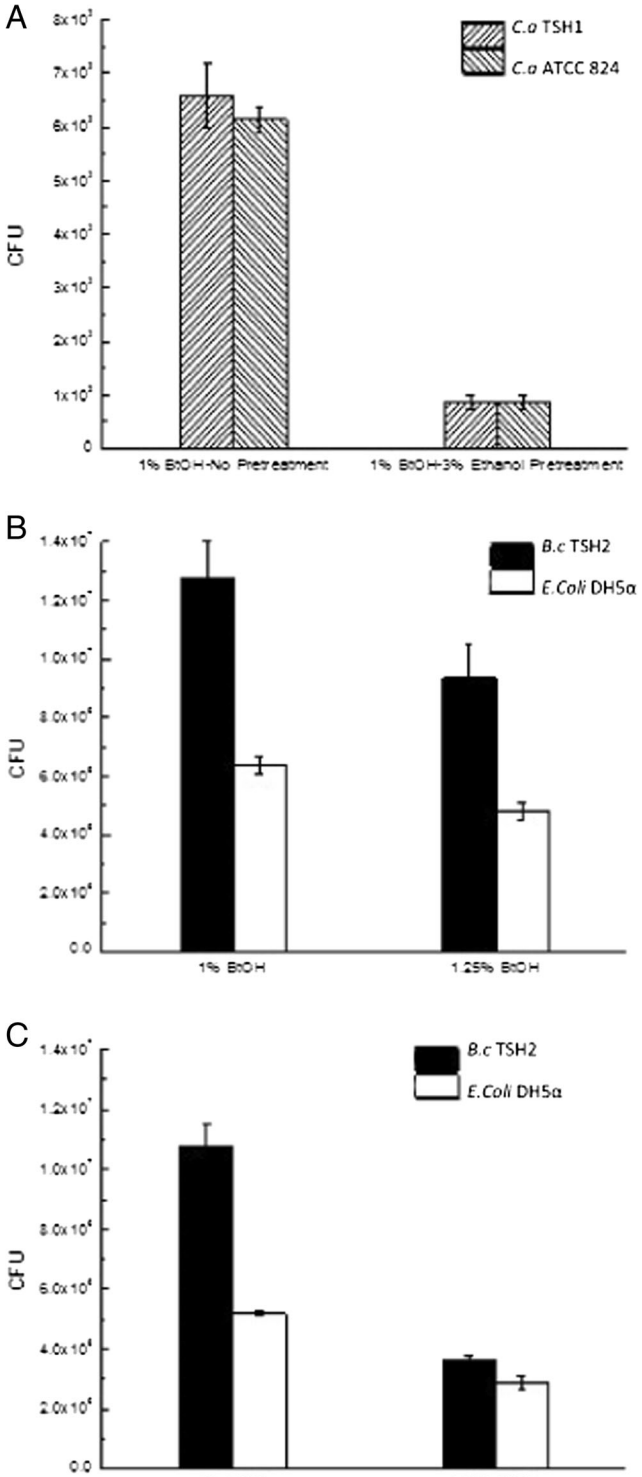

Fig. 6 Colony forming units (CFUs) in the presence of *n*-butanol. **a** CFU number of *C. acetobutylicum* ATCC 824 (*C.a* ATCC 824) and *C. acetobutylicum* TSH1 (*C.a* TSH1) without ethanol pretreatment; **b** CFU number of *E. coli* DH5 and *B. cereus* TSH2 (*B.c* TSH2) without ethanol pretreatment; **c** CFU number of *E. coli* DH5 and *B. cereus* TSH2 (*B.c* TSH2) with 3 % ethanol pretreatment

TSH06 was extraordinarily accelerated under non-anaerobic condition compared with that under anaerobic condition.

For non-anaerobic fermentation, Erlenmeyer flask was kept static without shaking, which was different from

conventional aerobic culture. However, cell inoculation and sample collection were both conducted in super clean bench. The flask was open, and air inevitably entered every 24 h. So, the fermentation condition was not anaerobic at all, even if it cannot be thought of as aerobic condition. TSH06 seeds were from one flask incubated non-aerobically. The cells were then inoculated in P2 medium. Three Erlenmeyer flasks were transferred into anaerobic chamber, and three were cultured in the ordinary incubator. The only difference was the presence of oxygen during fermentation. Since oxygen is harmful to *C. acetobutylicum* TSH1, it must be *B. cereus* TSH2 that consumes the oxygen and provides oxygen-free niche. However, *B. cereus* TSH2 itself cannot grow in P2 medium. How did it survive the whole fermentation process and play as a saver and accelerate the process at the same time? The two major factors of non-anaerobic fermentation were P2 medium and oxygen. P2 medium is a good substrate for *C. acetobutylicum* TSH1 but not for *B. cereus* TSH2. Non-anaerobic condition is suitable for *B. cereus* TSH2 but not for *C. acetobutylicum* TSH1. Best performance was obtained with co-culture of the two strains under the two conditions. These results demonstrated the potential advantage of symbiosis.

Alcohol tolerance of *C. acetobutylicum* TSH1 and *B. cereus* TSH2

To test butanol tolerance of the two isolated strains, CFUs were measured with butanol challenge. *C. acetobutylicum* ATCC 824 and *E. coli* DH5α were used as control. *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* TSH1 were cultured in P2 medium in anaerobic chamber at 37 °C. *E. coli* DH5α and *B. cereus* TSH2 were cultured in LB liquid medium aerobically at 37 °C. Cells were precultured with or without 3 % ethanol. *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* TSH1 were grown to OD_{600} of 1.0 for non-pretreated samples and 0.8 for pretreated samples and were used to plate on TYA solid medium with 1 and 1.25 % *n*-butanol, respectively. Colonies were measured after 48–72 h. The results were shown in Fig. 6a. CFU numbers were similar between *C. acetobutylicum* ATCC 824 and *C. acetobutylicum* TSH1. After pretreatment with ethanol, CFU number decreased to nearly half to that of un-pretreatment, and the numbers were still similar between the two strains. No colony appeared on TYA plate with 1.25 % butanol. It seemed hard for *C. acetobutylicum* to grow on solid medium supplemented with butanol. Original culture was even used for colony forming assay after several unsuccessful tries with diluted culture. *E. coli* DH5α and *B. cereus* TSH2 were grown to OD_{600} of 0.8 for non-pretreated samples and 0.6 for pretreated samples. Samples were used to plate on LB solid medium with 1 and 1.25 % *n*-butanol, respectively, after proper dilution. Colonies were measured after 12–18 h. CFU number was about twofold for *B. cereus* TSH2 compared with *E. coli* DH5α, except on 1.25 % *n*-butanol pretreatment with 3 % ethanol (Fig. 6b, c). Pretreatment with ethanol demonstrated decreased CFU numbers for both strains. So, *B. cereus* TSH2 demonstrated higher butanol tolerance than *E. coli* DH5α. Overall, CFU number of anaerobic strains was far less than that of aerobic strains. This difference can be attributed to the nature of anaerobe and aerobe. Aerobe possess grow advantage compared with anaerobe.

Discussion

In this study, butanol-producing symbiotic TSH06 was obtained, which can be cultured under non-anaerobic condition. TSH06 consisted of two strains, butanol-yielding, obligate anaerobic *C. acetobutylicum* TSH1 and anaerobic *B. cereus* TSH2 without butanol-producing ability. Butanol ratio reached more than 80 % in total solvent. Compared with pure *C. acetobutylicum* TSH1, TSH06 displayed advantage on butanol titer, yield and productivity. In addition, fermentation speed of TSH06 was faster under non-anaerobic condition than under anaerobic condition. TSH06 produce 12.97 g/L butanol from 60 g/L original glucose (54 g/L after sterilization) in 120 h. Operation of TSH06 is simple and time saving, with no need of special apparatus. All these advantages make TSH06 a potential application for industrialisation of biobutanol.

Oxygen is harmful to *Clostridium* strains once they are purified from their natural situation. Weizmann used a high ratio of loaded liquid and sterilisation to create anaerobic environment (Weizmann and Rosenfeld 1937). Endospore-forming *C. acetobutylicum* will enter dormancy by sporulation when the condition is undesirable, instead of dying. The endospore cell will turn to vegetative cell again once the condition is appropriate (O'Brien and Morris 1971). In symbiotic TSH06, facultative anaerobic *B. cereus* TSH2 produced a microanaerobic niche in the flask by consuming oxygen, which triggered the revival of anaerobic *C. acetobutylicum* TSH1. The latter obtained benefits from the coexistence, but *B. cereus* TSH2 seemed not. For *B. cereus* TSH2, it cannot grow in the fermentation medium and will be dormant by sporulation. However, the whole system was surely stable with a trace of *B. cereus* TSH2 under non-anaerobic condition, which was well illustrated in passage.

Stevens et al. (1987) used a mixed culture of *C. beijerinckii* and *B. cereus* to produce butanol from cheese whey. Higher butanol concentration was obtained in mixed-culture than pure *C. beijerinckii* culture. Unfortunately, the mixed-culture system did not catch much attention. Until recently, Trana et al. (2010) and Abd-Alla and Abdel-Wahab (2012) used *B. subtilis* in butanol fermentation as the auxiliary bacteria to consume oxygen in the system. Both of the authors emphasised that neither reducing agents nor N2 flushing were required anymore for butanol-producing anaerobic

Clostridium strain. Compared with those co-culture systems, TSH06 do not need anaerobic condition either. Moreover, the products were even significantly enhanced in symbiotic TSH06. Butanol concentration was only 10.39 g/L for pure *C. acetobutylicum* TSH1; however, it reached 13 g/L for symbiotic TSH06. Importantly, for TSH06 itself, fermentation speed was faster under non-anaerobic than that under anaerobic. So, *B. cereus* TSH2 does not only relieve anaerobic restriction but also enhance butanol production. These results also demonstrated clearly that co-culture is also a more efficient and simple strategy to enhance oxygen tolerance than metabolic engineering (Hillmann et al. 2008; Zhu et al. 2011; Dong et al. 2011).

The symbiotic character is also the basis of isolation of TSH06. Though single colony was picked from the TYA plate, the "apparent single colony" was not really a single strain. This could be attributed to the isolation condition. Non-strict anaerobic condition was employed during the whole procedure of butanol-producing strain isolation. TSH06 was thought once as a new type of strain according to the non-anaerobic trait. The above data illustrated clearly that *C. acetobutylicum* TSH1 was sensitive to oxygen. Once oxygen was exhausted, the spore will be active again. *C. acetobutylicum* TSH1 itself cannot even live through the microoxygen condition. So, the two strains were isolated together at the beginning and formed a dynamic system. When the medium and condition is suitable to *B. cereus* TSH2, *B. cereus* TSH2 gained an advantage and became the dominant cells, and vice versa. The profile of *C. acetobutylicum* TSH1 and *B. cereus* TSH2 is just like the situation in their natural habitat.

Symbiotic TSH06 produced a quarter more butanol than pure *C. acetobutylicum* TSH1. The high butanol product should be attributed to the presence of *B. cereus* TSH2. *B. cereus* TSH2 might increase butanol tolerance of *C. acetobutylicum* TSH1. In addition to tolerance of oxygen, *C. acetobutylicum* TSH1 got more benefit from symbiosis. However, the underlying mechanism is still unknown and worthy of exploring. Butanol ratio is also higher than the known reports of wild-type strains. It is even higher than gene *adc* disrupted mutant for inhibition of acetone production (Jiang et al. 2009). Since it was similar between *C. acetobutylicum* TSH1 and TSH06, the high butanol ratio should be a trait of *C. acetobutylicum* TSH1 but not *B. cereus* TSH2. *C. acetobutylicum* TSH1 is a close relative of *C. acetobutylicum* ATCC 824, DSM1731 and EA 2018 by alignment of several genes. Then, what makes the special butanol percentage of *C. acetobutylicum* TSH1? Omics study may help to solve this question. Anyway, high butanol proportion makes it easy for separation of butanol.

Symbiosis is universal. Microorganisms coexist side by side in original environment. They compete, fight and also cooperate with each other. Relationship of *Clostridium* and *Bacillus* is one of cooperation. *Bacillus* and *Clostridium* were also found coexisting in lignocellulase excretion (Kato et al. 2004) and hydrogen-yielding (Chang et al. 2008) system. *Bacillus thermoamylovorans* B5 can also shorten fermentation phase in hydrogen-producing co-culture compared with the pure *Clostridium* (Chou et al. 2011), but the yield was not improved. The facultative aerobic *Bacillus* guarantees the system with the ability of survival aerobically. This coexistence is more advantageous to anaerobic *Clostridium* by enlarging its living space, both for environment and substrate. *Clostridium* strains are obligate anaerobes, a fact difficult to overcome. Once it was isolated as a pure strain, it becomes critical for culturing. It cannot operate out of the anaerobic chamber or jar. Increasing numbers of strains are being isolated from their natural habitats. A purified strain makes it possible to study thoroughly and deeply. However, some superior properties are lost at the same time. It should be paid attention to. These results will also change the traditional conception of butanol fermentation. After one century of research, it is time for recurrence of co-culture of anaerobic *Clostridium* and aerobic *Bacillus*, as in natural habitat.

Acknowledgments This work was supported by the National Natural Science Foundation of China (no. 21176141), Tsinghua University Initiative Scientific Research Programme (no. 2012THZ02289), National Energy Administration (no. 20131448926), and Norway Statoil Petroleum AS.

Conflict of interest The authors declare that they have no competing interests.

References

Abd-Alla MH, Abdel-Wahab EE (2012) Production of acetone-butanol-ethanol from spoilage date palm (*Phoenix dactylifera* L.) fruits by mixed culture of *Clostridium acetobutylicum* and *Bacillus subtilis*. Biomass Bioenergy 42:172–178

Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl Environ Microbiol 53: 2854–2861

Chang J, Chou C, Ho C, Chen W, Lay J, Huang C (2008) Syntrophic co-culture of aerobic *Bacillus* and anaerobic *Clostridium* for bio-fuels and bio-hydrogen production. Int J Hydr Energy. 33:5137–5146

Chou C, Han C, Chang J, Lay J (2011) Co-culture of *Clostridium beijerinckii* L9, *Clostridium butyricum* M1 and *Bacillus thermoamylovorans* B5 for converting yeast waste into hydrogen. Int J Hydr Energy. 36(21):13972–13983

Dong H, Tao W, Zhu L, Zhang Y, Li Y (2011) CAC2634-disrupted mutant of *Clostridium acetobutylicum* can be electrotransformed in air. Lett Appl Microbiol 53:379–382

Ezeji TC, Qureshi N, Blaschek HP (2007) Bioproduction of butanol from biomass: from genes to bioreactors. Curr Opin Biotechnol 18(3): 220–227

Gheshlaghi R, Scharer JM, Moo-Young M, Chou CP (2009) Metabolic pathways of *clostridia* for producing butanol. Biotechnol Adv 27: 764–781

Hillmann F, Fischer RJ, Saint-Prix F, Girbal L, Bahl H (2008) PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. Mol Microbiol 68(4):848–860

Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H (2008) Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 77(6):1305–1316

Ishizaki A, Michiwaki S, Crabbe E, Kobayashi G, Sonomoto K, Yoshino S (1999) Extractive acetone-butanolethanol fermentation using methylated crude palm oil as extractant in batch culture of *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). J Biosci Bioeng 87:352–356

Jiang Y, Xu C, Dong F, Yang Y, Jiang W, Yang S (2009) Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. Metab Eng 11(4–5):284–291

Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50(4):484–524

Kato S, Haruta S, Cui Z, Ishii M, Igarashi Y (2004) Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. FEMS Microbiol Ecol 51:133–142

Lütke-Eversloh T, Bahl H (2011) Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr Opin Biotechnol 22(3):634–647

Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KL (2009) Engineering alternative butanol production platforms in heterologous bacteria. Metab Eng 11(4–5):262–273

O'Brien RW, Morris JG (1971) Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J Gen Microbiol 68(3):307–318

Papoutsakis ET (2008) Engineering solventogenic *clostridia*. Curr Opin Biotechnol 19:420–429

Stevens D, Alam S, Bajpai R (1987) Fermentation of cheese whey by a mixed culture of *Clostridium beijerinckii* and *Bacillus cereus*. J Ind Microbiol 3:15–19

Trana HTM, Cheirsilpa B, Hodgsonb B, Umsakulc K (2010) Potential use of *Bacillus subtilis* in a co-culture with *Clostridium butylicum* for acetone–butanol–ethanol production from cassava starch. Biochem Eng J 48:260–267

Weizmann C, Rosenfeld B (1937) The activation of the butanol-acetone fermentation of carbohydrates by *Clostridium acetobutylicum* (Weizmann). Biochem J 31(4):619–639

Wongwilaiwalina S, Rattanachomsri U, Laothanachareon T, Eurwilaichitr L, Igarashi Y, Champreda V (2010) Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. Enzym Microb Technol 47:283–290

Zhu L, Dong H, Zhang Y, Li Y (2011) Engineering the robustness of *Clostridium acetobutylicum* by introducing glutathione biosynthetic capability. Metab Eng 13(4):426–434

Zingaro KA, Papoutsakis ET (2013) GroESL overexpression imparts *Escherichia coli* tolerance to *i*-, *n*-, and 2-butanol, 1,2,4-butanetriol and ethanol with complex and unpredictable patterns. Metab Eng 15(1), 196–205