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Engineering *Bacillus subtilis* for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression

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Abstract In the present work, *Bacillus subtilis* was engineered as the cell factory for isobutanol production due to its high tolerance to isobutanol. Initially, an efficient heterologous Ehrlich pathway controlled by the promoter P_{43} was introduced into *B. subtilis* for the isobutanol biosynthesis. Further, investigation of acetolactate synthase of *B. subtilis*, ketol-acid reductoisomerase, and dihydroxy-acid dehydratase of *Corynebacterium glutamicum* responsible for 2-ketoisovalerate precursor biosynthesis showed that acetolactate synthase played an important role in isobutanol biosynthesis. The overexpression of acetolactate synthase led to a 2.8-fold isobutanol production compared with the control. Apart from isobutanol, alcoholic profile analysis also confirmed the existence of 1.21 g/L ethanol, 1.06 g/L 2-phenylethanol, as well as traces of 2-methyl-1-butanol and 3-methyl-1-butanol in the fermentation broth. Under microaerobic condition, the engineered *B. subtilis* produced up to 2.62 g/L isobutanol in shake-flask fed-batch fermentation, which was 21.3% higher than that in batch fermentation.

Keywords Isobutanol · *Bacillus subtilis* · Ehrlich pathway · Biosynthetic precursor pathway · Cell factory · Metabolic engineering

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Introduction

Isobutanol has been approved as a platform compound in food industry as well as in pharmaceutical and chemical industry for a long time (Karabektas and Hosoz 2009). Nowadays, it gains much interest as an ideal supplement or a sustainable replacement of gasoline due to its distinguished advantages, such as higher energy density and lower hygroscopicity compared with traditional biofuels (Atsumi et al. 2008; Nielsen et al. 2009).

The current demand for isobutanol is fulfilled by chemical synthesis from syngas, which requires high reaction temperature (350–500°C) and expensive catalyst such as palladium or rhodium (Carlini et al. 2003). Due to general environmental and economic issues, it is desirable to develop biosynthesis process to skip such energy-consumed and uneconomical steps. Vary from ethanol and butanol, which can be naturally produced by *Saccharomyces cerevisiae* and *Clostridia*, isobutanol biosynthesis is hampered owing to the lack of an economic native producer. Clostridial species, though they can produce *n*-butanol, are not ideal because of the relative lack of genetic tools to manipulate their metabolism, their slow growth, their sensitive to oxygen, and so on (Inui et al. 2008; Steen et al. 2008). In recent years, various synthetic metabolic pathways toward target compounds have been devised based on the progress of synthetic biology and metabolic engineering (Liu et al. 2010; Martin et al. 2003; Mukherji and van Oudenaarden 2009), which is holding great hopes for providing solutions to the unmet needs for humankind. In particular, the implementation of isobutanol biosynthesis in *Escherichia coli* is an excellent exemplar for new energy production by synthetic biological methodology (Atsumi et al. 2008; Zhanga et al. 2008).

However, further enhancement of isobutanol production by *E. coli* is impeded owing to its lower isobutanol-tolerant threshold (Smith et al. 2010). A more competent host is capable of enduring isobutanol at a high concentration; therefore, it is of great necessity in the long run. *Bacillus subtilis* is regarded as a great potential workhorse for isobutanol production because of its strong solvent-tolerant capacity (Fischer et al. 2008; Nielsen et al. 2009). Furthermore, it is also advantageous because of its native bio-safety, non-bias in codon usage, clear genetic background, and mature fermentative technology (Kunst et al. 1997; Wong 1995). So far to our knowledge, this organism has never been engineered for isobutanol production, and here in our present work, *B. subtilis* was engineered for isobutanol production as an attempt.

The production of desired chemicals can always be enhanced by overexpressing the precursor pathway, and this has been confirmed in many cases such as in riboflavin and antibiotic biosynthesis (Duan et al. 2010; Thykaer et al. 2010). The 2-ketoisovalerate (KIV) is the important precursor for isobutanol production, which is also shared by valine biosynthesis. KIV biosynthesis begins with the condensation of two pyruvate molecules to 2-acetolactate, which is catalyzed by acetolactate synthase (AlsS; EC 2.2.1.6). And then, KIV is produced through the consecutively catalysis by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ketol-acid reductoisomerase (IlvC; EC 1.1.86) and dihydroxy-acid dehydratase (IlvD; EC 4.2.1.9; Fig. 1). Previous researches have demonstrated that AlsS of *Corynebacterium glutamicum* is the key enzyme in KIV biosynthesis pathway because of feed-back inhibition (Leyval et al. 2003). However, the literature on AlsS of *B. subtilis* is rather limited to its enzymatic properties and regulatory factors (Atsumi et al. 2009; Holtzclaw and Chapman 1975; Renna et al. 1993), rarely mentioned its role in KIV biosynthesis pathway. Therefore, it raises our interest to investigate the position of AlsS in precursor and isobutanol biosynthesis pathways of the engineered *B. subtilis*.

In this work, a recombinant *B. subtilis* for isobutanol biosynthesis was first constructed. Additionally, the important role of AlsS on isobutanol biosynthesis was elucidated, and strain improvement was performed by overexpressing a de novo biosynthetic KIV precursor pathway. Genetic stability, metabolic profile, and fermentation strategy of the engineered *B. subtilis* were also investigated.

Materials and methods

Regents

All enzymes were purchased from Fermentas Co., Ltd (Glen Burnie, MD, USA). All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were ordered from Invitrogen Biotechnology Co., Ltd (Carlsbad, CA, USA), and DNA sequencing was served by BGI (Beijing, China).

Bacterial strains, plasmids, and genetic manipulation

The strains and plasmids used in this work are listed in Table 1. *E. coli* JM109 was used to propagate all plasmids. All *Bacillus* strains used were derived from *B. subtilis* 168, which was used as the wild type (WT). All DNA manipulations were carried out using standard techniques (Sambrook and Russell 2001). The transformation of *E. coli* and *B. subtilis* were performed by heat shock (Sambrook and Russell 2001) and the competent cell method (Anagnostopoulos and Spizizen 1961), respectively. Primers used for plasmid construction are listed in Table 2.

To engineer isobutanol biosynthesis recombinants, plasmids pHPL(01–04) were constructed. First of all, plasmid pHPL01 was created by blunting the large *Bsm*I-*Nco*I fragment of pHP13 and then auto-ligated. P_{43} promoter (GenBank GeneID, 140119566) and *alsS* gene (GenBank GeneID, 936852) were amplified with two pairs of primers P_{43}-F/P_{43}-R and *alsS*-F/*alsS*-R by using *B. subtilis* genomic DNA (GenBank accession no. NC_000964) as PCR template. The P_{43} PCR product digested with *Hind*III and *Pst*I was cloned into pHPL01 plasmid cut with the same enzymes to obtain pHPL02. pHPL03 was constructed by amplifying *adh*2 (GenBank GeneID, 171020) of *S. cerevisiae* from its genomic DNA (GenBank accession no. NC_001140) with *adh*2-F/*adh*2-R, digesting the PCR product with *Bam*HI and *Sma*I and ligating into pHPL02 cut with the same enzymes. Similarly, pHPL04 was constructed by amplifying *kivd* (GenBank GeneID, 51870501) of *Lactococcus lactics* from its genomic DNA with *kivd*-F/*kivd*-R, digesting the PCR product with *Bam*HI and *Pst*I and ligating into pHPL03 cut with the same enzymes.

Plasmid pDGMPKA was used to construct the isobutanol production mutant *B. subtilis* BSUL02. Before obtaining pDGMPKA, a multiple cloning site (MCS) cut from pUCMCS with *Hind*III and *Eco*RI was cloned into the plasmid pDG1730 cut with the same enzymes, creating pDGM. Then PKA (P_{43}::*kivd-adh2*) was amplified with a pair of primers PKA-F and PKA-R using pHPL04 as a PCR template. The PCR product was digested with *Sph*I and *Sal*I and ligated into pDGM cut with the same enzymes to obtain pDGMPKA.

Plasmids pHPL(05–09) were used to investigate the biosynthetic precursor pathway for isobutanol production. The *alsS* PCR product digested with *Xma*I and *Pvu*I was cloned into pHPL02 and pHPL04 cut with the same enzymes, respectively, creating pHPL05 and pHPL06. To

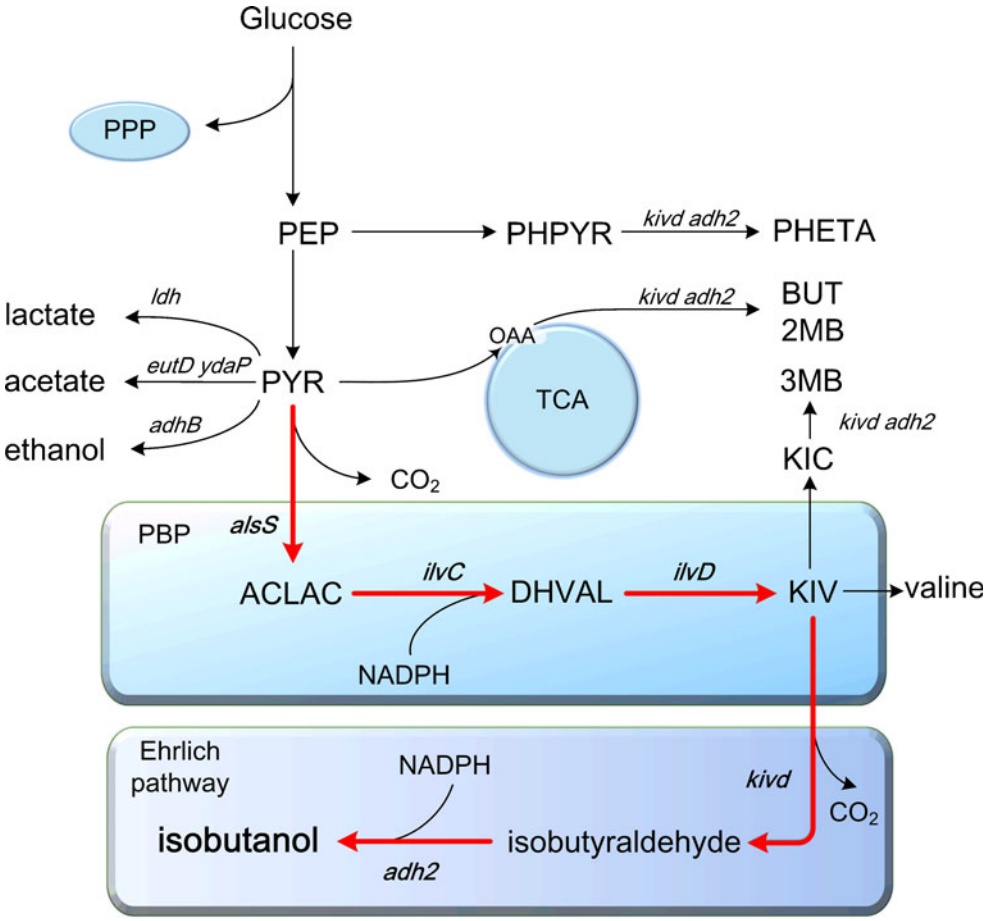


Fig. 1 Schematic representation of heterologous pathway leading to isobutanol formation in *B. subtilis* (gene names are shown in *italics*). *Red arrows* and *bold gene names* represent the reconstructed isobutanol biosynthesis pathway and the enzymes involved, respectively. *PEP* phosphoenolpyruvate, *PYR* pyruvate, *OAA* oxaloacetate, *ACLAC* acetolactate, *DHVAL 2,3-dihydroxy-3-methylbutanoate*, *KIV* 2-ketoisovalerate, *KIC* 2-ketoisocaproate, *BUT butanol*, *2MB* 2-methyl-1-butanol, *3MB* 3-methyl-1-butanol, *PHPYR* phenylpyruvate, *PHETA* 2-phenylethanol, *NADPH* nicotinamide adenine dinucleotide phosphate (reduced form), *PPP* pentose phosphate pathway, *TCA* citrate cycle, *BPP* biosynthetic 2-ketoisovalerate precursor pathway. Genes *ldh*, *eutD*, *ydaP*, *adhB*, *alsS*, *ilvC*, *ilvD*, *kivd*, and *adh2* encode lactate dehydrogenase, phosphate acetyltransferase, pyruvate oxidase, alcohol dehydrogenase, acetolactate synthase, ketol-acid reductoisomerase, dihydroxy-acid dehydratase, keto-acid decarboxylase, and alcohol dehydrogenase

clone *ilvC* (GenBank GeneID, 1019254) and *ilvD* (GenBank GeneID, 1019249), *C. glutamicum* genomic DNA (GenBank accession no. NC_006958) was used as a template with two pairs of primers *ilvC*-F/*ilvC*-R and *ilvD*-F/*ilvD*-R, respectively. The *ilvD* PCR product was digested with *Kpn*I and *Pst*I and cloned into pHPL02 cut with the same enzymes to obtain pHPL07. Similarly, pHPL08 was obtained by digesting the *ilvC* PCR product with *Bgl*II and *Pvu*I and ligating into pHPL07 cut with the same enzymes. Finally, pHPL09 was obtained by digesting *alsS* PCR product with *Afe*I and *Xho*I and ligating into pHPL08 cut with the same enzymes.

The *B. subtilis* recombinants were selected by antibiotic screening. The mutants were further confirmed by observing amylase halo, PCR verification, and DNA sequencing with a pair of primers *amyE*-F and *amyE*-R.

Medium and cultivation

Unless stated otherwise, all *E. coli* and *B. subtilis* strains were cultured in Luria–Bertani (LB) at 37°C. For stepwise construction of isobutanol and biosynthetic precursor pathways, the cultures were grown in 5 mL LB medium in 16× 200 mm test tubes. To prepare seed cultures, stains stored at −80°C were incubated on LB agar plate for 18 h, and one colony of recombinants was selected and cultivated at 240 rpm. To study the isobutanol biosynthesis behavior and metabolic profile, the seed cultures were diluted 1:100 into 20 mL culture medium (LBGSM-I or LBGSM-II) and cultivated under microaerobic condition (cultured in the screw-cap flask at 240 rpm). LBGSM-I medium was composed of LB medium with 20 g/L glucose, 100 mM potassium phosphate buffer (pH 7.0), and 1,000 dilution of

Table 1 Strains and plasmids used in this study

Name	Relevant genotype	Source or reference
Strains		
B. subtilis 168	Wide-type strain, *trpC2*	BGSC
C. glutamicum ATCC 13032	Auxotrophic for biotin	ATCC
S. cerevisiae S288C	*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1*	ATCC
L. lactics ATCC19435	*Lactococcus lactis* subsp. *lactis* (Lister) Schleifer et al. deposited as *Streptococcus* sp.	CMGCC (Schleifer et al. 1985)
E. coli JM109	*recA1,endA1,gyrA96,thi-1,hsdR17,supE44,relA1, Δ(lac-proAB)/F'[traD36,proAB+,lacIq,lacZΔM15]*	TransGen Biotech (Beijing, China)
BSUL01-1	P_{43}::*adh2*, Em^r	This study
BSUL01-2	P_{43}::*kivd-adh2*, Em^r	This study
BSUL01-3	P_{43}::*kivd-adh2-alsS*, Em^r	This study
BSUL02-1	*ΔamyE*::(P_{43}::*kivd-adh2-Spc*^{r}), Spc^r	This study
BSUL02-2	*ΔamyE*::(P_{43}::*kivd-adh2-Spc*^{r}), P_{43}::*alsS*, Spc^r, Em^r	This study
BSUL02-3	*ΔamyE*::(P_{43}::*kivd-adh2-Spc*^{r}), P_{43}::*ilvD-ilvC*, Spc^r, Em^r	This study
BSUL03	*ΔamyE*::(P_{43}::*kivd-adh2-Spc*^{r}), P_{43}::*ilvD-ilvC-alsS*, Spc^r, Em^r	This study
Plasmids		
pHP13	*B. subtilis–E. coli* shuttle plasmid; Cm^r, Em^r	BGSC (Haima et al. 1987)
pHPL01	*B. subtilis–E. coli* shuttle plasmid; Em^r,	This study
pHPL02	Em^r; P_{43}::	This study
pHPL03	Em^r; P_{43}::*adh2*	This study
pHPL04	Em^r; P_{43}::*kivd-adh2*	This study
pHPL05	Em^r; P_{43}::*alsS*	This study
pHPL06	Em^r; P_{43}::*kivd-adh2-alsS*	This study
pHPL07	Em^r; P_{43}::*ilvD*	This study
pHPL08	Em^r; P_{43}::*ilvD-ilvC*	This study
pHPL09	Em^r; P_{43}::*ilvD-ilvC-alsS*	This study
pDG364	*B. subtilis* integration plasmid; Amp^r, Cm^r	BGSC (Karmazyn-Campelli et al. 1992)
pUCMCS	Amp^r; pUC18 with multiple cloning sites (MCS) from pDG364	This study
pDG1730	*B. subtilis* integration plasmid; Amp^r, Spc^r	BGSC (Guérout-Fleury et al. 1996)
pDGM	Amp^r, Spc^r; MCS	This study
pDGMPKA	Amp^r, Spc^r; P_{43}::*kivd-adh2*	This study

BGSC Bacillus Gentic Stock Center, *ATCC* American Type Culture Collection, *CGMCC* China General Microbiological Culture Collection Center

Trace Metal Mix A5 (Atsumi et al. 2008); LBGSM-II medium was prepared by adding another 20 g/L glucose into LBGSM-I medium. For isobutanol fermentation, experiments were carried out in the 1-L flasks under microaerobic condition with a 200-mL work volume and 1% inoculation. LBGSM-II medium was used for batch fermentation, while fed-batch fermentation was initiated with LBGSM-I medium and followed by a glucose addition after 18 h cultivation in order to keep the total glucose concentration equal to that in LBGSM-II medium. The pH values were adjusted to 7.0 by adding 10 M NaOH at 18 h. Antibiotics were added appropriately (erythromycin 0.5 μg/mL, ampicillin 100 μg/mL, and spectinomycin 100 μg/mL).

Isobutanol toxicity tolerance assays

To assay isobutanol toxicity tolerance, *B. subtilis* and *E. coli* were cultivated in LB medium at 37°C, while *C. glutamicum* was cultured in GCIII medium at 30°C (Menkel et al. 1989). When the optical density at 600 nm (OD_{600}) reached to 0.4~0.6, some of the cultures were properly diluted and spread evenly onto corresponding agar plates with varied isobutanol concentration (0%, 0.5%, 1.0%, 1.5%, and 2.0%) for the viable rate calculation after 24 h incubation, and some of the cultures were directly inoculated into the corresponding liquid medium to cultivate for the first 2 h, followed by adding isobutanol at a cascading concentration stated above and kept on cultivating for another 22 h to measure OD_{600} for

the relative growth calculation. The viable rate and the relative growth were defined as below:

$$\text{the viable rate} = \frac{\text{the number of colonies on isobutanol contained agar plate}}{\text{the number of colonies on non-isobutanol contained agar plate}} \times 100\%$$

$$\text{the relative growth} = \frac{\text{OD}_{600}\text{ of cultures in isobutanol contained medium}}{\text{OD}_{600}\text{ of cultures in non-isobutanol contained medium}} \times 100\%$$

Enzymatic activity assays in crude extracts

The crude cell extract was prepared as described previously (Leyval et al. 2003). Quantification of ADH activity was performed by using the method developed by Smith et al. (2010). Total protein concentrations were measured by Bradford (1976) assay.

Real-time PCR analysis of *alsS* transcription in recombinant *B. subtilis*

The relative mRNA abundance of *alsS* of different recombinants was measured by real-time PCR (RT-PCR; Bio-Rad, USA) to further validate its key role in isobutanol biosynthesis pathway. Total RNA was extracted by Trizol reagent (Qiagen, Germany) and then treated with DNase I (Fermentas, USA). Gene *gapA* (GenBank Gene ID, 938627) was used as internal control. Primers used were prefixed with RT and listed in Table 2. Data analysis was performed according to the comparative C_T method (Livak and Schmittgen 2001).

Table 2 Oligonucleotides used in this study

Primer name	Sequence[a] 5′→3′
P_{43}-F	GGTAAAGCTTGCGGCTTCCTTGTAGA
P_{43}-R	CTCTCTGCAGCATGTGTACATTCCTCTC
kivd-F	GAGGCTGCAGATGTATACAGTAGGAGATTA
kivd-R	GCGTGGATCCTTATGATTTATTTTGTTC
adh2-F	CTTAGGATCCAAGGGGTGTCCAACATGTCTA TTCCAGAAACT
adh2-R	TGGGAGCCCGGGTTATTTAGAAGTGTCAACA ACGTAT
ilvC-F	TTTGCAGATCTGGAGGATTTTCTCCCATGGC
ilvC-R	ATATACGATCGCTCGAGAGCGCTCCAGACAAT
ilvD-F	TACTAGGTACCGGAGGAAGCGCATCATGATC
ilvD-R	ATACCCTGCAGAGATCTACAGGCCAAGACATG
alsS-F	CATGACCCGGGAGCGCTAGCATTGCTTAGT GGAG
alsS-R	AATCTCGATCGCTCGAGAATCTGGCTCACAGG
PKA-F	CGGCAAGATCTGTTGTGTGGAATTGTGAGCGG
PKA-R	TCGTAGTCGACGACGTTGTAAAACGACGGCC
amyE-F	CGATTCAAAACCTCTTTACTG
amyE-R	CCATTTAGCACGTAATCAAAG
RT-*alsS*-F	GGGTGCCTCTAACTTGGC
RT-*alsS*-R	TGCACGGATCACGTTTCC
RT-*gapA*-F	CAAACAAGGCGTTGAAAT
RT-*gapA*-R	GCGCCAGCTTCTAAGTGT

[a] Underline stands for the restriction site

Genetic stability assays

Genetic stability and assessment were performed as described (Bi et al. 2009). The strain retaining both erythromycin and spectinomycin resistance was cultured for ten generations to evaluate the stability of isobutanol biosynthesis.

Analytical methods

Metabolite identification by gas chromatography–mass spectrum

Alcohol compounds produced by our strains were identified by gas chromatography (GC)–mass spectrum (6890N GC System and 5975C mass selective detector, Agilent Technologies, USA) with a DB-5ms capillary column (30 m, 0.25-mm internal diameter, 0.25-μm film thickness, Agilent Technologies, USA). The supernatant was harvested by centrifugation at 10,000×*g* for 10 min, and alcohols were extracted by vortex mixing 500 μL supernatant with 300 μL GC standard grade chloroform for 2 min and then centrifuged for 5 min to obtain the sample-containing chloroform for analysis. A 1-μL sample was injected for analysis. The operating conditions were performed as described previously (Atsumi et al. 2008).

Metabolite quantified by gas chromatography–flame ionization detector and high-performance liquid chromatography

The alcohol compounds were quantified by gas chromatography–flame ionization detector (7890A G3440A, Agilent Technologies, USA) equipped with PEG-20M capillary column (30 m, 0.32-mm internal diameter, 0.25-μm film

thickness, Shanghai Kechung Chromatograph Instruments Co., Ltd, China). The oven temperature was programmed to maintain at 60°C for 1 min, then increased at a rate of 10°C/min to 110°C, further at a rate of 30°C/min to 230°C, and maintained for 2 min. Injector and detector temperatures were both kept on 230°C. Other fermentation products were determined by high-performance liquid chromatography (1200, Agilent Technologies, USA) equipped with a Zorbax SB-C18 column (250×4.6 mm, Agilent Technologies, USA) and a UV detector (210 nm). The mobile phase of 5 mM H_2SO_4 at a 0.3-mL/min flow rate was adopted. The working temperature of the column was kept on 30°C.

OD_{600}, cell dry weight, and glucose concentration measurement

The OD_{600} was measured using a microplate reader (Model 550, Bio-Rad, USA). Cell dry weight (CDW) was measured by filtering the cell suspension with a filter and drying the filter paper and cells to a constant weight for 24 h at 105°C. The linear relationship between CDW and OD_{600} was obtained with a conversion factor of 0.325. Glucose concentration in culture broth was determined enzymatically by a bioanalyzer (SBA-40C, Shandong, China).

Results

Isobutanol toxicity tolerance assay

B. subtilis can withstand 1-butanol very well (Fischer et al. 2008), while little was known about its isobutanol tolerance. In the present study, the isobutanol toxicity tolerance of *B. subtilis* was first evaluated by exposing the organism to this solvent at different concentrations. Additionally, the isobutanol production host *E. coli* (Atsumi et al. 2008) and the latest reported *C. glutamicum* (Smith et al. 2010) were selected as control. For agar plate cultivation, both *B. subtilis* and *C. glutamicum* grew well when isobutanol concentration was less than 1%, and the viable rate was 56.0% and 55.7%, respectively, higher than 30.4% of *E. coli*. *B. subtilis* exhibited the highest toxicity tolerance under high isobutanol concentration (2%), and the viable rate was 2.5-fold of *C. glutamicum* and 20-fold of *E. coli*, respectively. The similar results were observed in liquid cultivation. Though *C. glutamicum* and *E. coli* showed a little higher relative growth (94.6% and 89.5%, respectively) than *B. subtilis* (82.5%) at 0.5% isobutanol concentration, *B. subtilis* exhibited a higher relative growth value in the presence of 2% isobutanol, which was 1.9-fold of *C. glutamicum* and 3.8-fold of *E. coli*, respectively (Fig. 2). These results showed a beneficial characteristic of *B. subtilis* of being a cell factory for isobutanol production.

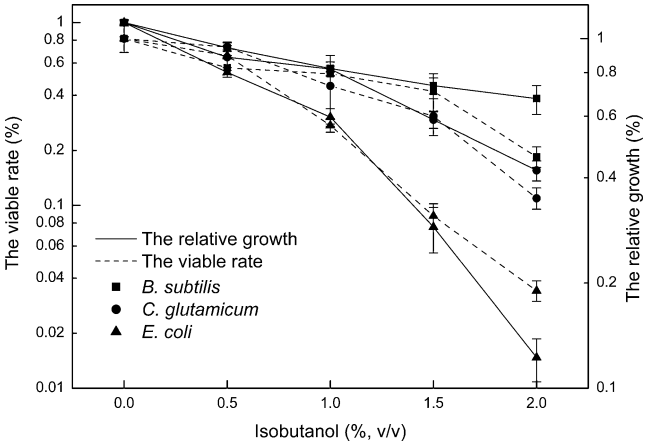


Fig. 2 Comparison of isobutanol toxicity tolerance of *B. subtilis*, *C. glutamicum*, and *E. coli* by exposure to isobutanol. Data were expressed as average values and standard deviations of three parallel studies

Engineering *B. subtilis* for isobutanol production by constructing an efficient heterologous Ehrlich pathway

For the initial attempt to engineer the *B. subtilis* cell factory for isobutanol production, the pathway capable of converting KIV to isobutanol should be constructed first. Atsumi et al. (2009) showed that the *E. coli* strain overexpressing *alsS* of *B. subtilis* without keto-acid decarboxylase (KDC; EC 4.1.1.72) was still able to produce isobutanol, demonstrating that AlsS functions as both 2-ketoisovalerate decarboxylase (Kdc activity) and AlsS (Als activity). Additionally, the enzyme activity of native alcohol dehydrogenase (ADH; EC 1.1.1.1) to isobutyraldehyde was 54.50± 2.44 U/mg in WT in the present work. However, the fact that isobutanol could not be detected in WT might ascribe to the inadequate ADH activity for isobutanol biosynthesis. Therefore, *adh2* from *S. cerevisiae* was expressed heterologously under the control of a strong promoter P_{43} of *B. subtilis* to ensure the efficiency. The ADH activity of the resulted recombinant was 94.54±1.98 U/mg, approximately doubled than that of WT, confirming that both P_{43} and heterogeneous ADH function well. Unfortunately, the fact that isobutanol could not be detected in this recombinant probably ascribed to the insufficient Kdc activity of native AlsS. This assumption was confirmed by further overexpression of *kivd* from *L. lactics*. As we expected, the resulted strain BSUL01-2 could accumulate 0.69±0.05 g/L isobutanol (Table 3).

To increase the genetic stability of recombinant, the heterologous Ehrlich pathway was then inserted into the chromosome of WT (Fig. 3). By using the integration vector pDGMPKA (Table 1) with a neutral site *amyE* for the aim of eliminating the polar effect caused by gene disruption and insertion, the resulted strain BSUL02-1 produced 0.57±0.06 g/L isobutanol (Table 3).

Table 3 Isobutanol biosynthesis of different recombinant *B. subtilis*

B. subtilis strains[a]	BSUL01-1	BSUL01-2	BSUL01-3	BSUL02-1	BSUL02-2	BSUL02-3	BSUL03
alsS overexpression	–	–	+	–	+	–	+
Isobutanol, g/L	ND	0.69±0.05	1.35±0.14	0.57±0.06	1.24±0.27	0.63±0.13	1.58±0.21

ND not detected

[a] Bacteria were cultured for 30 h in LBGSM-I medium under 37°C at a rotation of 240 rpm; data were expressed as average values and standard deviations of three parallel studies

BSUL02-1 with single copy of Ehrlich pathway (integration into the chromosome) produced less isobutanol than BSUL01-2 with multi-copy (without integration; Table 3). Simultaneously, BSUL02-1 showed a longer exponential phase and lower specific growth rate (Fig. 4). That was probably due to the imbalance of KDC, ADH, and AlsS accumulation in vivo, as KDC and ADH were expressed constitutively while the synthesis of AlsS was regulated by many factors (Renna et al. 1993). For that speculation, *alsS* was constitutively overexpressed and the resulted recombinants BSUL02-2 and BSUL01-3 produced almost equivalent isobutanol (Table 3).

Strain improvement by overexpressing a de novo biosynthetic KIV precursor pathway

It was observed that isobutanol production was unexpectedly enhanced after overexpression of AlsS (Table 3). AlsS of *C. glutamicum* has been identified as the key enzyme in valine biosynthesis (Leyval et al. 2003), whereas in *B. subtilis*, little was reported about its role in isobutanol biosynthesis that shard the same KIV precursor with valine biosynthesis. For this limitation, AlsS, IlvC, and IlvD involved in the biosynthetic precursor pathway were investigated. Compared with the parental strain BSUL02-1, BSUL02-3 with IlvD and IlvC overexpression increased isobutanol production to 1.1-fold, while BSUL02-2 with only AlsS overexpression elevated the production to 2.2-fold. BSUL03 harboring the de novo biosynthetic precursor pathway (P_{43}::*ilvD-ilvC-alsS*) accumulated 1.58±0.21 g/L isobutanol, which was 2.8-fold of BSUL02-1 and 2.5-fold of BSUL02-3, respectively, whereas only 1.3-fold of BSUL02-2 (Table 3). These results implied that AlsS was an important enzyme for KIV precursor biosynthesis. Then it was validated by analyzing the relative mRNA abundance of *alsS* with RT-PCR. Taking *gapA* gene as internal standard, it was clearly observed that the transcription levels of *alsS* in BSUL02-2 and BSUL03 were much higher than those in BSUL02-1 and BSUL02-3 (Fig. 5) and presented an approximately equal level. These results further confirmed that it was AlsS rather than IlvC or IlvD that played a more significant role in isobutanol biosynthesis.

Genetic stability analysis of the engineered BSUL03

Genetic stability is important for engineered strains in industrial production. Analysis showed that plasmid pHPL08 transformant was quite stable in BSUL03. The percentages of cells cultivated without antibiotic selection pressure retained erythromycin resistance were as follows: 100±2.2% after five generations and 97.5±9.4% after ten generations. In addition, all the progeny still retained spectinomycin resistance with the percentage of 100±3.2% after ten generations indicated a stable homologous recombination in BSUL03. Strain with both erythromycin and spectinomycin resistance was cultured for ten generations, and a stable isobutanol production of 1.73±0.11 g/L was observed. These observations suggested that BSUL03 was genetic stable for isobutanol production.

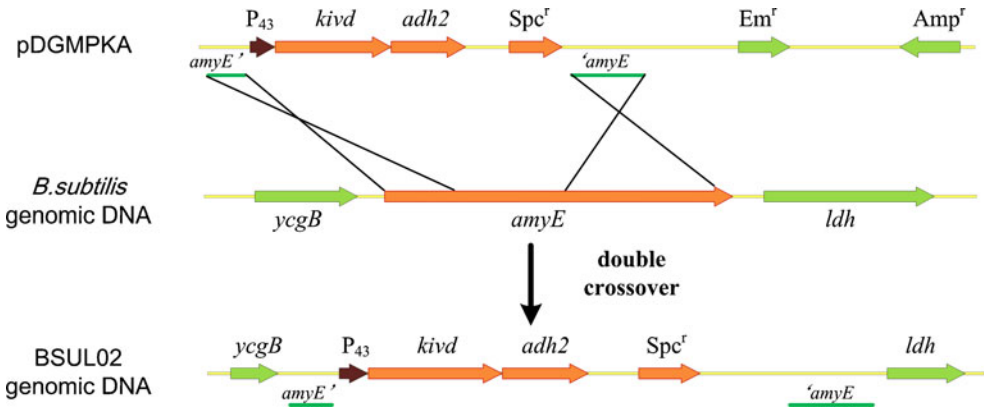


Fig. 3 Schematic diagram of the construction of recombinant BSUL02-1. The heterologous Ehrlich pathway (P_{43}::*kivd-adh2*) with a spectinomycin cassette was inserted into *amyE* locus of *B. subtilis* chromosome

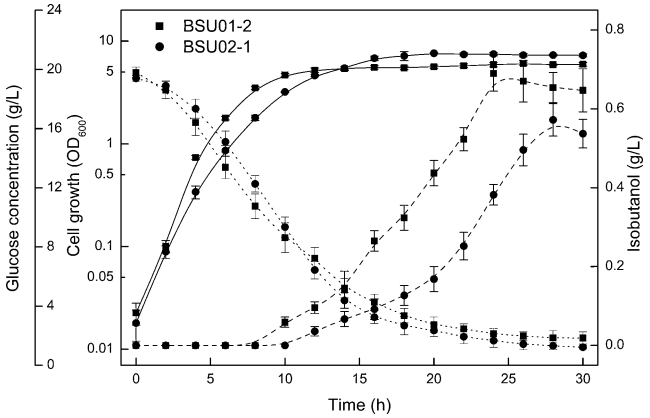


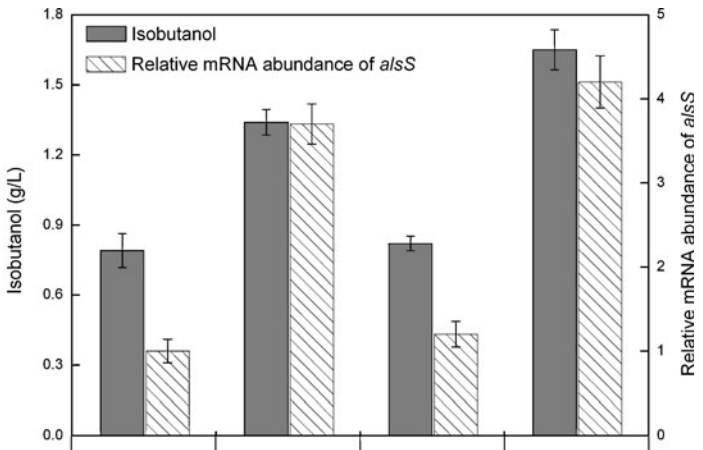
Fig. 4 Comparison of the isobutanol biosynthesis and cell growth between BSUL01-2 and BSUL02-1 in LBGSM-I medium cultured for 30 h. The *solid line*, *dash line*, and *dot line* represent cell growth (OD_{600}), isobutanol production, and glucose concentration, respectively. Data were expressed as average values and standard deviations of three parallel studies

Isobutanol biosynthesis and metabolic profile analysis of recombinant *B. subtilis*

To investigate the isobutanol biosynthesis and metabolic profile, WT and recombinants were cultured under microaerobic condition. All the recombinants showed less biomass than WT after 40 h growth, especially the strains with the Ehrlich pathway genes encoded in multi-copy plasmid. Recombinants with only *alsS* overexpression (BSUL01-3 and BSUL02-2) enhanced isobutanol production by 95.7% and 132.5% than their parental strains. BSUL03 harboring the de novo biosynthetic precursor pathway produced the highest isobutanol production of 2.02±0.26 g/L (Table 4). In addition to isobutanol, other branched-chain higher alcohols were analyzed. 2-Phenylethanol (PHETA) existed in all recombinants, while 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) were only detected with trace amount in *alsS* overexpressed recombinants. Acetate, lactate, and ethanol were detected as major by-products in fermentation broth. In contrast to the stable production of ethanol in all strains, the production of acetate and lactate varied from strains with *alsS* overexpression or not. For BSUL01-2 and BSUL01-3, the decrease of acetate and lactate were 109% and 14%, respectively, while the values were 34% and 24% for BSUL02-1 and BSUL02-2. Therefore, acetate and lactate could be regarded as potential reconstruction targets for further strain improvement (Ji et al. 2008).

Isobutanol fermentation of BSUL03 by different fermentation strategies

To select a suitable fermentation process for the best isobutanol producer BSUL03, both batch and fed-batch fermentation strategies were investigated. The experiments were carried out in 1 L shake flasks under microaerobic condition. In fed-batch fermentation, though the cell grew with a slower specific rate of 0.16 h^{-1}, isobutanol was produced at a faster production rate of 0.086 g/L/h;



	BSUL02-1	BSUL02-2	BSUL02-3	BSUL03
ΔamyE::(P_{43}::*kivd-adh2-Spc*^{r})	+	+	+	+
pHPL05 (P_{43}::*alsS*)		+		
pHPL08 (P_{43}::*ilvD-ilvC*)			+	
pHPL09 (P_{43}::*ilvD-ilvC-alsS*)				+

Fig. 5 Effect *alsS* in biosynthetic precursor pathway on isobutanol production and its transcriptional analysis. Both isobutanol production and the relative mRNA abundance of *alsS* were represented by *column*. *Plus symbols* denote overexpression of the indicated gene (s) or pathway(s) in recombinant *B. subtilis*. Data were expressed as average values and standard deviations of three parallel studies

Table 4 Isobutanol production and metabolic profile of *B. subtilis* strains under microaerobic condition

B. subtilis strains[a]	P_{43}::*kivd-adh2* integration	*alsS* overexpression	CDW g/L	Alcohols (g/L)					Other by-products	
				Isobutanol	Ethanol	PHETA	2MB	3MB	Acetate	Lactate
WT	−	−	2.48±0.08	ND	1.22±0.17	ND	ND	ND	8.27±0.43	6.13±0.28
BSUL01-2	−	−	1.99±0.04	0.93±0.12	1.35±0.21	1.38±0.11	ND	ND	8.75±0.31	6.21±0.26
BSUL01-3	−	+	2.15±0.02	1.82±0.21	1.46±0.14	1.15±0.24	0.04±0.01	0.11±0.01	4.18±0.29	5.44±0.31
BSUL02-1	+	−	2.31±0.04	0.77±0.16	1.17±0.09	1.16±0.17	ND	ND	4.69±0.17	7.03±0.23
BSUL02-2	+	+	2.34±0.07	1.79±0.17	1.24±0.15	1.08±0.13	0.03±0.00	0.07±0.01	3.50±0.21	5.67±0.19
BSUL03	+	+	2.35±0.06	2.02±0.26	1.21±0.18	1.06±0.15	0.05±0.00	0.08±0.01	3.15±0.17	4.23±0.12

ND not detected

[a] Bacteria were cultured for 40 h in LBGSM-II medium under 37°C at a rotation rate of 240 rpm; data were expressed as average values and standard deviations of three parallel studies

comparatively, the values were 0.27 h^{-1} and 0.073 g/L/h in batch fermentation, respectively. Besides, the biomass of fed-batch fermentation was 14.8% higher than that of batch fermentation. At the end of fermentation, the maximal isobutanol titer was up to 2.62±0.19 g/L at 48 h, which was 21.30% higher than that in batch fermentation at 42 h (Fig. 6). In addition, 8.12±1.11 g/L residual glucose, 5.32 g/L acetate, and 6.35 g/L lactate were detected in batch fermentation broth, which was 1.3-fold, 1.7-fold, and 1.3-fold of those in fed-batch fermentation, respectively (Fig. 6). The higher concentration of residual glucose, acetate, and lactate might explain the lower isobutanol production rate and final titer due to the lower conversion ratio in batch fermentation.

Discussion

Isobutanol attracts intensive interest as a promising biofuel for its notable advantages and has recently been biosynthesized in the engineered *E. coli* (Atsumi et al. 2008). However, *B. subtilis* with a much higher solvent-tolerant capacity can be considered as a preferable cell factory for isobutanol production.

The isobutanol toxicity tolerance of *B. subtilis*, *E. coli*, and *C. glutamicum* was compared in the present work, and results showed that *B. subtilis* exhibits a higher tolerance to isobutanol than the others (Fig. 2). The reasons might be as follows: On the one hand, the thick cell wall of *B. subtilis* protects the cell against the external stresses such as pressure and hyperosmotic shock (Hayhurst et al. 2008), which is important for a cell to keep alive; on the other hand, a large group of nonspecific stress proteins, which are mediated by the general stress σ^B factor and induced together by environmental stress, possess an essential protective function on cell survival (Marles-Wright et al. 2008; Petersohn et al. 2001). These protective mechanisms are crucial for *B. subtilis* to stand against high isobutanol concentration, which will be a distinct advantage of *B. subtilis* once isobutanol titer reaches the threshold of the present producers.

Previous and our present work demonstrated the existence of ADH encoded by *adhB* in WT (Kunst et al. 1997) and its enzyme activity to isobutyraldehyde (54.50±2.44 U/mg), respectively. Hence, isobutanol can be biosynthesized theoretically since the native AlsS possesses Kdc activity to convert KIV to isobutyraldehyde (Atsumi et al. 2009). However, the failure of isobutanol detection in WT and recombinant BSUL01-1 with *adh2* overexpression (Table 3) might ultimately ascribe to the low affinity of the native AlsS to isobutyraldehyde, whose K_m value is 300 mM in vitro (Atsumi et al. 2009), approximately 158 times of that of *L. lactic* (1.9 mM; de la Plaza et al. 2004). Therefore, the

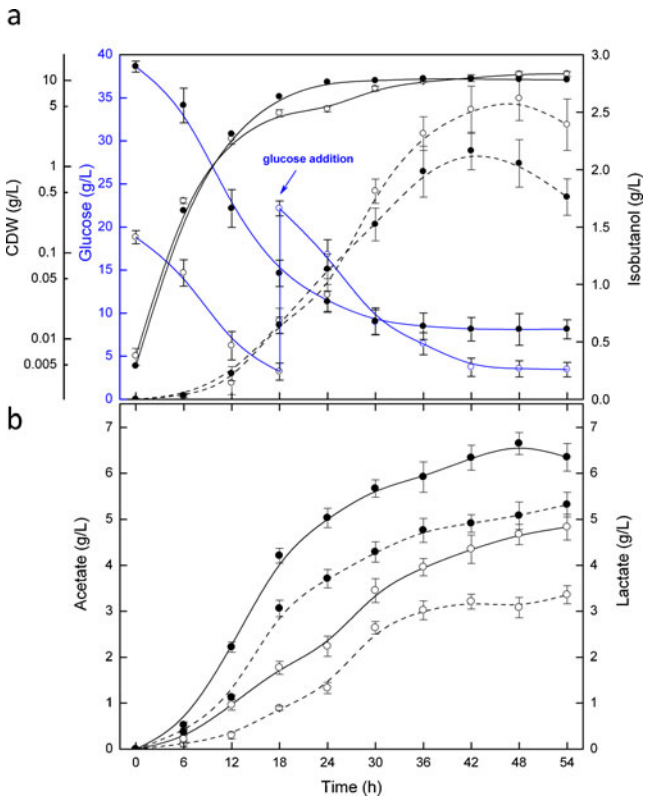


Fig. 6 Isobutanol biosynthesis and metabolic profile of BSUL03 by two different fermentation strategies. *Black circle* batch fermentation, *white circle* fed-batch fermentation; **a** *black line* CDW, *dash line* isobutanol, *blue line* glucose; **b** *black line* lactate, *dash line* acetate. Data were expressed as average values and standard deviations of three parallel studies

heterologous Ehrlich pathway was constructed by putting *kivd* and *adh2* in series under the control of P_{43}, which is a well-characterized strong promoter of *B. subtilis* and has been used to strengthen the gene expression (Wang and Doi 1984). By introducing this heterologous Ehrlich pathway into *B. subtilis*, isobutanol could be detected in all recombinants. Simultaneously, it was interesting to find that the strains with single copy of the Ehrlich pathway showed a longer exponential phase but lower isobutanol production in comparison with those with multi-copy (Table 3). This is plausible as AlsS responsible for KIV formation is synthesized in detectable quantities only in stationary-phase cultures (Renna et al. 1993), and it was observed that 80–90% of isobutanol was accumulated in this period in the present work (Fig. 4), whereas the discrepancies between strains just mentioned were vanished after overexpressing the *alsS* gene constitutively (Table 3), further indicating a concernful influence of AlsS on isobutanol biosynthesis. Additionally, it was worth noting that isobutanol production was greatly enhanced beyond our expectation in *alsS* overexpressed recombinants, which dropped the hint of the significant role of AlsS for isobutanol biosynthesis.

In view of the potential importance of AlsS stated above, it was attractive to investigate the AlsS and the other enzymes involved in KIV precursor pathway. Overexpressing *alsS*, *ilvC*, and *ilvD* individually and together showed that IlvC and IlvD had little influence on isobutanol production (Table 3), which highlighted the importance of AlsS. Moreover, the obvious dependence of isobutanol production on *alsS* expression level showed by transcriptional analysis further validated the dominated role of AlsS in the KIV precursor biosynthesis pathway of *B. subtilis* (Fig. 5), which consisted with the results elucidated in several microorganisms (Eggeling et al. 1987; Leyval et al. 2003). To enhance isobutanol production, a de novo biosynthetic KIV precursor pathway was constructed. It was important to retain the native AlsS because of its high affinity to pyruvate (Gollop et al. 1990). Additionally, IlvC and IlvD from *C. glutamicum* were also selected as they exhibit the highest affinities for their respective substrate (the K_m values of both of them are around 1 mM) among all the bacteria so far as we known at pH 7.3 and pH 8.0, respectively, which are close to the optimal pH for native AlsS (Chang et al. 2009; Leyval et al. 2003). However, it has to point out that the present work did not investigate the enzyme activities of native IlvC and IlvD. Recombinant BSUL03 harboring the de novo biosynthetic KIV precursor pathway produced 2.8-fold isobutanol of its parent BSUL02-1 (Table 3), which demonstrated the great importance of precursor supply on production enhancement of the desired chemicals (Thykaer et al. 2010). Inspired by these results, other approaches such as promoting the enzyme activity by directed evolution can be adopted to enhance precursor biosynthesis and further improve isobutanol production.

The metabolic profiles of recombinants were investigated under microaerobic condition. In addition to isobutanol, both 2MB and 3MB were detected with trace amount, whereas to our surprise, at least 1.06 g/L PHETA was existed in every recombinant (Table 4). PHETA is widely used in food and cosmetic industry, and its biosynthesis mainly relies on yeast at present (Etschmann and Schrader 2006). Since other PHETA producers are less productive, such as *E. coli*, which produces at most 0.89 g/L PHETA by adding 8 g/L phenylpyruvate precursor (Atsumi et al. 2008), and *C. glutamicum* possesses a weak biosynthesis ability with the production of 0.11 g/L PHETA (Smith et al. 2010), *B. subtilis* is considered as a promising PHETA producer succeeding to yeast. By employing *B. subtilis*, PHETA can be biosynthesized as the main product after further strain development, or be produced as a major byproduct that can be easily separated from isobutanol. It was also observed that ethanol production in all *B. subtilis* strains was stable, a litter higher than PHETA (Table 4). Previous work

demonstrated that ethanol, which is formed by acetyl-CoA come from pyruvate, was another product in addition to the major anaerobic fermentation products acetate and lactate (Cruz-Ramos et al. 2000; Nakano et al. 1997). These results indicated that during isobutanol fermentation, *B. subtilis* underwent the mixed acid–butanediol fermentation style. Acetate and lactate were produced at a high concentration in fermentation broth under microaerobic condition, which was not only a waste of carbon source but also a hindrance for cell growth. Usually, it is preferred to increase the conversion of carbon substrates to desired products by deleting the mixed acid biosynthesis pathway (Jian et al. 2010; Unrean et al. 2010). In *B. subtilis*, it was reported that 80% acetate could be reduced by *alsS* overexpression with the desired product increased by 50% (Zhu et al. 2007). Lactate was also demonstrated as an important competitor with acetolactate to pyruvate available (Romer-Garcia et al. 2009). In accordance with those facts, our results (Table 4) indicated a redistribution of carbon flux by *alsS* overexpression, which led to an increase of pyruvate availability to KIV biosynthesis. This strategy might also provide a positive signal for *menp1* promoter and coordinate the activation of TCA cycle and formation of the respiratory chain (Qin and Taber 1996) and further influenced the primarily energy metabolism that was important for isobutanol biosynthesis because of its dependence on adenosine triphosphate and NADP(H) supply. It was observed that lactate did not decrease so sharply as acetate in the present work, which probably owing to the discrepancy of K_m value of different enzymes to pyruvate: 3.0 mM of lactate dehydrogenase (Garvie 1980) and 13.6 mM of AlsS (Atsumi et al. 2009). Therefore, acetolactate was greatly competed by lactate for pyruvate consumption, and it was helpful to increase isobutanol production by inactivating lactate biosynthetic pathway. For instance, isobutanol production was increased by ~25% to 4.9 g/L by constructing an isobutanol-producing *C. glutamicum* in a *ΔpycΔldh* background (Smith et al. 2010).

The suitable fermentation process was also surveyed by employing different strategies in this work. Compared with batch fermentation, the isobutanol production was increased by 21.3% and up to 2.62 g/L in fed-batch fermentation, while a 36.6% and 23.9% reduction of acetate and lactate were observed simultaneously. A slower decrease of pH value was observed in fed-batch fermentation than that in batch fermentation (data not shown, the pH value at 18 h was 6.17 in fed-batch fermentation while 4.82 in batch fermentation), as by intermittent substrate feeding, the cell growth could be well regulated to avoid the broth over-acidification. Therefore, the possible reasons for the higher isobutanol production were: (1) the avoidance of over-acidification kept a relative favorable pH value for the enzymes responsible for isobutanol production and therefore raised the efficiency of bio-catalysis and (2) the reduction of mixed acid resulted in the increase of pyruvate availability to KIV and therefore enhanced isobutanol production. Further, isobutanol production can be effectively improved by different fed-batch operational strategies such as constant substrate feeding rate and pH control (Gordillo et al. 1998; Mu et al. 2009).

Despite some achievements have been made in engineering *B. subtilis* as a promising cell factory for isobutanol production, there are still many difficulties to be dealt with, such as how to devise an efficient biosynthesis pathway and how to direct the carbon flux. On the one hand, it is important to explore and screen enzymes with high catalytic activity to further increase the isobutanol biosynthesis efficiency. For instance, *adh2* from *S. cerevisiae* is used to encode ADH for branched-chain higher alcohols production (Atsumi et al. 2008; Connor and Liao 2008), while Atsumi et al. (2010) confirmed that ADH encoded by *yqhD* or *adhA* from *E. coli* is better than *adh2* for isobutanol production. On the other hand, it is necessary to decrease the by-products as much as possible, which can prevent the inhibition to cell growth and direct the carbon flux toward the target effectively. By blocking ethanol formation, Ji et al. (2010) improved 2,3-butanediol to 130 g/L in *Klebsiella oxytoca*, and this was also worked for isobutanol production improvement (Atsumi et al. 2008). At present, with the development of metabolic engineering and computational biology (Hädicke and Klamt 2010; Leonard et al. 2009), strains can be improved by rational design, which is based on the genome-scale network that contains comprehensive relationships of genes, proteins, and reactions. Over the last few years, a number of approaches have been proposed for the phenotype simulation of microorganisms under different environmental and genetic conditions. An in silico aided metabolic engineering strategy is popular because of its accurate identification of target genes that lead to production enhancement, which saves lots of time to obtain an ideal strain. Strain improvement by in silico design based on genome-scale network has been well applied to *E. coli* (Feist et al. 2010) and *S. cerevisiae* (Brochado et al. 2010), which sets a good example to further optimize the *B. subtilis* cell factory for isobutanol production.

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