Metabolic Engineering of Bacillus subtilis for 2.3-BDO production by introducing an exogenous NADPH/NADP+ regeneration system

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Research

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Abstract

Background: Generally, glucose is transformed into pyruvate from glycolysis before the target products acetoin and 2,3-butanediol (2,3-BDO) are formed. Pentose Phosphate Pathway (PPP) is an inefficient synthetic pathway for pyruvate production from glucose in Bacillus subtilis. Previously, it was found that engineered PPP in B. subtilis unbalanced NADH and NADPH regeneration systems and affected acetoin and 2,3 -BDO production.

Results: In this study, metabolic engineering strategies were proposed to redistribute carbon flux to 2,3-BDO via reconstructing intracellular cofactors regeneration systems. Firstly, extra copies of glucose dehydrogenase (GDH)and an exogenous NADPH-dependent 2,3-BDO dehydrogenase (TDH) were introduced into the GRAS strain B. subtilis 168 to introduce an exogenous NADPH/NADP + regeneration system and broaden 2,3-BDO production pathway. It was found that overexpressing the NADPH/NADP + regeneration system effectively improved 2,3-BDO production and inhibited NADH-dependent by-products accumulation. Subsequently, the disruption of lactate dehydrogenase (encoded by ldh) by insertion of the transcriptional regulator ALsR, essential for the expression of alsSD (encoding two key enzymes for the conversion of pyruvate to acetoin) in B. subtilis, resulted in the recombinant strain in which alsSD was overexpressed and the pathway to lactate was blocked simultaneously. On fermentation by the result engineered strain, the highest 2,3-BDO concentration increased by18.43%, while the titers of main byproducts acetoin and lactate decreased by 22.03% and 64%, respectively.

Conclusion: In this study, it shows that engineering PPP and reconstructing intracellular cofactors regeneration system could be an alternative strategy in the metabolic engineering of 2,3-BDO production in B. subtilis .

Background

2,3-butanediol （2.3-BDO）is an important platform compound and it has important applications in industry [1-3]. As a potential environmentally friendly fuel, the heating value of 2.3-BDO is 27.2 kJ/g [4] comparing with ethanol 29.055 kJ/g and methanol 22.081 kJ/g [5], it can mixed with gasoline in any proportion. Due to the shortage of fossil fuels, the production of 2,3- BDO is catching more attentions [1].

The microorganisms, which can produce 2,3-BDO, are *Serratia marcescens* [6], *Klebsiella* [7], *Bacillus polymyxa* [8], *B. amyloliquefaciens* [9], etc. Compared with other known 2,3-BDO producing strains, *B. subtilis* is also superior for its GRAS status that meets safety regulations for industrial-scale fermentation [10]. In the metabolic pathway of 2, 3-BDO, glucose is transformed into pyruvate from glycolysis before 2,3-BDO is formed and then, pyruvate is converted into acetoin under the acetolactate synthase(ALS) and acetolactate decarboxylase(ALDC)（Figure 1）[11, 12]. 2,3-BDO is synthesized under the 2,3-BDO dehydrogenase with the acetoin as substrate [13].

In recent years, production of 2, 3-BDO by microorganisms has been greatly developed by regulating NADH regeneration system. Yang et al. improved 2,3-BDO production by 22.7% in *B.amyloliquefaciens* via rebuilding NADH/NAD^+ regeneration system by co-overexpression of Glyceraldehyde-3-Phosphate Dehydrogenase /2,3-butanediol Dehydrogenase [14], and increased 2,3-BDO titer by 25.5% in *B. subtilis* via introducing an extra NADH regeneration system and disrupted NADH oxidase [15]. Fu et al. successfully enhanced the 2,3-BDO titer by 13.6% with the introduction of transhydrogenase to improve the NADH yield from NADPH in *B. subtilis* [16]. Dai et al. simultaneously increased 2,3-BDO titer by the higher NADH/NAD^+ratio in *Paenibacillus polymyxa* with additional vitamin C in the fermentation medium [17]. These regulatory strategies were all based on regulating NADH levels. In this study, we payed more attentions to regulate a different cofactor NADPH levels and tried to improve 2, 3-BDO titer by reconstructing a NADPH/$NADP^+$ regeneration system.

Pyruvates could be produced via two pathways: the Glycolysis Pathway (EMP) and Pentose Phosphate Pathway（PPP）. In the EMP, one glucose produces two pyruvates accompanying with two molecules of NADH [1]. While, through the PPP, pyruvate is produced accompanying with NADPH production [18]. In our previous study, it was found that acetoin could be enhanced by engineering the PPP pathway and disturbing the coenzyme balance of NADH/NADPH[19]. In this study, we tried

to introduce extra copies of glucose dehydrogenase(GDH)[20] and an exogenous NADPH-dependent 2,3-BDO dehydrogenase(TDH)[21] into the GRAS strain *B. subtilis 168* to introduce an exogenous NADPH/$NADP^+$ regeneration system and observed their effects on 2,3-BDO fermentation.

Result

Engineering of pentose phosphate pathway and its effects on glucose fermentation to 2,3-BDO in *B. subtilis*

Pyruvate is the precursor of acetoin [11, 12]and a key intermediate product in glucose metabolism [12]. There are main two pathways for production pyruvate in microorganisms: EMP and PPP. Pyruvate can be produced via EMP and PPP in microorganisms. GDH (encoded by *gdh*), one of the key enzymes in the gluconate production, catalyzes the conversion of glucose to gluconate accompanying with NADH and NADPH. Gluconate is converted into pyruvate through the pentose phosphate pathway [19, 22]. In this work, GDH under different strength promoters P_{bdhA} [23], P_{pac}, P_{Spac} [24] and P_{HpaII} were separately overexpressed into *Bacillus subtilis*, and the resultant strains were BM1, BM2, BM3, BM4, respectively.

The fermentation results showed that overexpression of GDH in engineered strains repressed the cell growth and 2,3-BDO production and extended the fermentation period in medium containing 100 g/L glucose（Table 3）. The yield of byproduct acetoin was increased by 17.6-37.8%, but 2,3-BDO, lactate and ethanol decreased by 15.1-22.3%, 15.2-23.3%, 32.3-37.6% respectively. It might indicate that overexpression of GDH disturbed the NADH and NADPH regeneration systems and repressed 2,3-BDO fermentation.

Introducing an exogenous NADPH-dependent 2, 3-butanediol dehydrogenase to construct the NADPH/$NADP^+$ regeneration system in engineered *B. subtilis* strains

Overexpression of GDH increased the level of NADPH and decreased NADH levels which repressed the conversion of acetoin to 2,3-BDO. To enhance the carbon flux to 2, 3-BDO , we tried to introduce an exogenous NADPH-dependent 2,3-BDO dehydrogenase[21] (coding gene *tdh* in *Clostridium autoethanogenum DSM 10061*（into engineered *B.subtilis* to construct an extra NADPH/$NADP^+$ regeneration system with help of GDH, and observed their effects on 2,3-BDO fermentation.

In eukaryotes, poly（A） tails are stretch adenine bases in the 3' end of RNA, and it was found that when the poly(A) tails become short enough, the mRNA could be degraded. It was also reported that the different of poly(A) tails had a great effect on the stability of mRNA of the enzyme, which plays a key role in its overproduction process [25-27].

In this study, to improve the efficiency of carbon flow from acetoin to 2,3-BDO, poly (A) tails (AAATTT, TTTAAATTT, AAATTTAAATTT, TTTTTTAAATTT and TTTTTT)were separately added to the 3' end of the *tdh* to improve its transcription abundance and TDH production in *B. subtilis*, and the resultant strains named BM6, BM7, BM8, BM9 and BM10, respectively. The results were showed in Figure 2. The transcription abundance of tdh in strains BM6, BM7, BM8 and BM9 were all decreased, compared with strains BM5 without any poly (A/T) tails, and the lower transcription abundance resulted in lower TDH activities in strains BM6, BM7, BM8 and BM9. However, compared with strains BM5, the transcription abundance of TDH in strain BM10 increased by 85 %, which resulted in the TDH activity reached to 180.35 mu/mg and increased by 32.6 %.

Subsequently, the *TDHR5* and *gdh* with different promoters were co-overexpressed in *B. subtilis* 168 resulting in recombinant strains BM11, BM12, BM13 and BM14 (Tale 3). It was observed that fermentation times were shortened significantly, the NADPH levels were decreased, and the yields of 2.3-BDO were increased but the acetoin titer was decreased when compared with GDH solely overexpressed strains BM2, BM3, BM4 and BM5 (Tale 3). The strain BM12 (GDH under promoter P_{pac})

showed the highest titer of 2,3-BDO which was increased by 39.64% while the acetoin titer was decreased by 36.82 %, which suggested that excessive NADPH was used for the synthesis of 2,3-bdo, and more acetoin was catalyzed to 2,3-bdo.

When compared with the parental strain *B.subtilis 168*, it could be found that introduction of an efficient exogenous NADPH/$NADP^+$ regeneration system (co-overexpressing GDH and TDH) in strain BM12 increased 2,3-BDO titer by 14.01% and total amount of acetoin and 2,3-BDO by 3.05% while decreased the titers of NADH-dependent by-products lactate and ethanol by 12.82% and 23.65% , respectively. It indicated that we successfully redistributed the carbon flux to 2,3-BDO pathway by regulating cofactors regeneration systems in *B. subtilis*.

Redistributing the carbon flux to 2,3-BDO by overexpressing the transcriptional regulator ALsR and blocking the pathway to lactate

ALsR is a LysR-type transcriptional regulator , which could regulate the *alsSD* operon, encoding the key enzyme (ALS and ALDC), catalyzing pyruvate to acetoin [28, 29]. To redistribute more carbon flux to 2,3-BDO pathway, ALsR under promoter P_{srfA} [30] was overexpressed by integrated into the chromosome of BM12 at *ldh*(encoding lactate dehydrogenase , LdhA) locus, resulting in strain BM15. The multiple copies of *alsr* improved the activities of als (from 1.0 U/mg to 1.75U/mg) and aldc (from 0.51 U/mg to 0.75U/mg) by 75% and 47.05% and decreased the activity of LdhA by 89 % when compared with strain BM12. Shown in Table 3.

As shown in Figure 3A, the cell density has no obvious change among BM0, BM12 and BM15. However, the introduction of extra copies of *alsR* and disrupt of *ldh* in strain BM15 resulted in faster glucose consume rate, increased 2,3-BDO by 2.5% and decreased acetoin and lactate titer by 10.34% and 59.03% compared with strain BM12.

Furthermore, when compared with parental strain *B.subtilis 168*, through all above metabolic engineering strategies in strain BM15, we shortened the fermentation time from 72 hours to 70 hours, increased 2, 3-BDO titer and total titer of acetoin and 2,3-BDO by 18.35% and , while decreased the titers of acetoin and NADH-dependent by-products lactate by 30.64% and 64.28% respectively(Figure3).

Discussion

As fossil fuels become increasingly scarce, bio-production of 2,3-BDO is attracting increasing attention [1]. Generally, sugars are converted into pyruvate mainly from glycolysis before the target product 2,3-BD is formed [11]. As known that pyruvates could be also produced via PPP. However, it was found that PPP is an inefficient synthetic pathway for pyruvate production from glucose in *B. subtilis* [19]. GDH (encoding by *gdh*) is one of the key enzymes catalyzes the conversion of glucose to gluconate which enter PPP [19] . Thus, we tried to overexpress GDH in *B. subtilis* to enhance the metabolic efficiency of PPP and broaden the pathways from glucose to pyruvate. It was found that overproduction of GDH in *B. subtilis* increased NADPH levels and acetoin accumulation but decreased NADH levels and 2,3 -BDO production, and extended the duration. The disturbed levels of cofactors should be responsible for inhibited carbon utilization rate. Lower levels of NADH decreased NADH-dependent products 2,3-BDO, lactate and ethanol production. Insufficiency NADH availability inhibited transformation of acetoin to 2, 3-BDO, so acetoin accumulated more.

In 2,3-BDO biosynthesis pathway, it contains a series of redox reactions which participate in regulating the NADH/NAD^+ ratio in bacteria [14]. Overproduction of GDH in *B. subtilis* disturbed NADH and NADPH levels and repressed 2,3-BDO fermentation. Thus, we need introduce an exogenous NADPH/$NADP^+$ regeneration system to rebalance cofactors regeneration system in *B. subtilis* and improve 2,3-BDO fermentation. NADPH-dependent 2.3-BDO dehydrogenase could be found in anaerobic microorganisms such as *Clostridium beijerinckii* [31] and *Clostridium. autoethanogenum* [21].

Some cofactors metabolic engineering strategies have been employed to improve 2, 3-BDO production and repress by-products production by manipulating NADH levels. To improve the NADH yield from NADPH in *B. subtilis*, Fu et al. introduced

a transhydrogenase and successfully enhanced the 2,3-BDO titer by 13.6% [16]. In previous study, we manipulated NADH levels in *B. subtilis* by introducing an extra NADH regeneration system (Formate dehydrogenase and formate) while simultaneously disrupting the NADH oxidase and *ldh* genes, which increased the 2,3-BDO titer by 25.5%, with a concomitant decrease acetoin (by 76.4%) and lactate (by 80.5%) accumulation [15]. Inspired by these results, we tried to set up a $NADPH/NADP^+$ regeneration cycle to redistribute the carbon flux to 2,3-BDO with NADPH as cofactor. As it was expected, when a NADPH-dependent 2.3-BDO dehydrogenase was successfully co-overexpressed with GDH in *B. subtilis*, it significantly increased the 2,3-BDO production and sharply decreased acetoin and NADH-dependent by-products lactate and ethanol formation, and shortened the fermentation duration. It indicated that we successfully redistributed the carbon flux to 2,3-BDO pathway and improve the consumption rate of glucose by reconstructing the $NADPH/NADP^+$ regeneration systems in *B. subtilis*.

In bacterial metabolism, ethanol, acetoin, lactate, and other end-products are also produced during 2,3-BD fermentation [32]. Therefore, 2,3-BD biosynthesis must compete with multiple pathways for the pyruvate and NADH resources, which remains prohibitively low for commercial production of 2,3-BDO. To improve the yield of 2,3-BDO, overproduction of key enzymes involved in the 2,3-BDO pathway and blocking the unwanted by-products pathways are all alternative strategies. ALsR acts as the regulatory gene of *alsSD* operon encoding ALS and ALDC, which is responsible for acetoin biosynthesis [28, 29]. Some researchers had tried to overexpressing ALS and ALDC to enhance the acetoin production. However, the higher activities of ALS and ALDC suppressed the cell growth, the acetoin yield was not significantly increased [23, 33]. Zhang et al. attempted to control ALS and ALDC by moderately enhancing the expression of AlsR, and successfully improved the yield of acetoin by 62.9% in *B. subtilis* [23]. The deletion of *adhE* (encoding alcohol dehydrogenase) and *ldh* gene (encoding lactate dehydrogenase) could block the carbon flux toward ethanol and lactate biosynthesis increase the available NADH for 2,3-BD formation [34-36]. In this study, to redistribute the carbon flux to 2,3-BDO ,we tried to regulate the expression level of ALsR using the promoter P_{srfA}, which is self-induced and transcribes at the middle and late stage of fermentation [30]. The fused gene fragment P_{srfA}-ALsR was inserted into the location of *ldh* in the *B. subtilis* genome, which resulted in the recombinant strain in which ALsR was overproduced and *ldh* was disrupted simultaneously. It was found that the lacking of *ldh* and extra copies of ALsR in *B. subtilis* resulted in more carbon to flow into the 2, 3-BDO synthesis pathway, and increased the 2, 3-BDO titer by 18.35% and decreased the yields of by-products AC, lactate and ethanol by 22.03 %, 64.28 % and 25.80% respectively. The fermentation duration was shortened to 70 h.

Conclusion

In this study, we proposed an alternative strategy to regulate the carbon flux toward 2,3-BDO by introducing an exogenous $NADPH/NADP^+$ regeneration system, overexpressing ALsR under the promoter P_{srfA} and disrupting *ldh* gene simultaneously. The production of 2,3-BDO was increased, and the by-products production of acetoin, and lactate and ethanol were decreased significantly, which indicated that engineering of NADPH coenzyme cycles could improve 2,3-BDO production and suppress the by-products accumulation.

Materials And Methods

Bacterial strains, primers, and plasmids

All bacterial strains used are described in Table 1. All primers and plasmids used in this study are described in Table 2

Culture conditions

Strains were cultured in Luria-Bertani medium at 37 °C on a rotary shaker at 180 rpm, if necessary, add 50 mg / L of Kana Magnesium, 100 mg/L ampicillin or 3% bleomycin into the medium.

For 2,3-BDO fermentation, the fermentation medium included 100 g/L glucose ,5 g/L yeast extract 20 g/L corn syru and 2 g/L urea, pH 6.8. The fermentation medium was sterilized at 121 °C for 20 min. The seed was cultured in the LB at 37 °C on a rotary shaker at 180 rpm for 12 h, 3 mL seed was inoculated into 50 mL fermentation medium at 180 rpm and 37 °C.

The construction plasmids and Strains

The promoter P_{bdhA} and gene *gdh* was amplified from the *B. subtilis 168* genome by the primers P1 and P2, P9 and P10 respectively, the promoters Ppac and Pspac were synthesized in Genewize company. It was obtained by PCR by primers P3 and P4, P5 and P6. Promoters P_{bdhA}, P_{pac}, P_{Spac}, P_{HpaII} and *gdh* genes connected together by overlap extension PCR(SOE PCR) [37], then insert to the site of *EcoRI* in pMA5. For the construction of pMA5-P_{HpaII}-*TDHRX* (X was from 1to 5), the gene *tbdh* was synthesized in Genewize company, *TDH, TDHR1, TDHR2, TDHR3, TDHR4, TDHR5,* design with were obtained by PCR with primers p11 and p12, p13, p14, p15, p16, p17 respectively, and then ligated into the vector pMA5 at *NdeI* and *BamHI* restriction sites. For the pMA5-P_{HpaII}-*TDHR5*-Ppac-*gdh* construction, firstly construct pMA5-P_{HpaII}-*TDHR5*, then inserted *gdh* under different promoters insert to the site of *EcoRI* of pMA5. All ligation between genes and vector were used Clon Express II One Step Cloning Kit (Vazyme Biotech in NanJing, China).

Gene *alsR* and the promoter P_{srfA} were amplified by PCR by primers P26 and P27, P24 and P25, respectively. The gene *zeo* was amplified from p7z6 by primers P18 and P19. *ldhA*-F (1000 bp upstream *ldhA* gene in genome) and *ldhA*-R (1000 bp downstream *ldhA* gene in genome) were obtained by primers P20 and P21, P22 and P23, respectively. Then, *ldhA*-R, *ldhA*-F,*zeo*, P_{srfA} and *alsR* were connected together through SOE-PCR.

The method of transform plasmid or DNA fragments into *Bacillus subtilis 168* reference the method described by Vojcic et al. [38]

Enzyme assays

To determine acetoin reductase (ACR) activity, bacteria was cultured overnight, the cells were collected by centrifugation for 5 min at 8000 rpm, and washed with 0.1 M pH 7.0 phosphate buffer for three times. ACR activity was assayed spectrophotometrically by monitoring the change in absorbance at 340 nm corresponding to the reduction of NADPH at 37 °C in a total volume of 1 mL 0.1 M phosphate buffer containing 0.2 mM NADPH and 50 mM acetoin. The enzyme activity unit was defined that the amount of enzyme required to consume 1 μmol/L NADPH per minute. Total protein concentrations were determined according to the Bradford method.

To determine the activity of LdhA, the total volume of 1mL 0.1M phosphate buffer（pH 7.2）containing 0.2 mM NADH and 10 mM sodium pyruvate was assayed spectrophotometrically by monitoring the change in absorbance at 340 nm corresponding to the reduction of NADH at 37 °C, the enzyme activity unit was defined that the amount of enzyme required to consume 1 μmol/L NADH per minute.

The enzyme activities of ALS[39]and ALDC[40] asssys were performed according to published procedures.

Quantitative real-time PCR (qRT-PCR)

To determine the transcription abundance of *tdh*, cells cultured 8-10 h were harvested at room temperature and immediately frozen in liquid nitrogen. the total RNA of bacteria was extracted using Bacteria RNA Extraction Kit (Vazyme Biotech in NanJing, China), and then reverse-transcribed into cdna, which was used as the template for rt-pcr. Expression levels of different *tbdh* genes were measured with primers p28 and P29. The 16S rRNA gene was used as the internal standard with primers p30 and P31.

Detection of metabolites

The cell mass density was determined from the OD at 600 nm in an ultraviolet (UV)-visible spectrophotometer (UNICO UV-2000 spectrophotometer, Shanghai, China). Glucose, 2,3-BD and acetoin were analyzed using high performance liquid chromatograph (HPLC) system (Agilent Corp., USA) with RID detector. The column and mobile phases were Hi-Plex Ca (4.6 x 250 mm) (Agilent, USA) and ultrapure water at 0.4 ml/min, respectively. Lactate and ethanol were detected by HPLC used column HPX-87H (BioRad) with a mobile phase of 2.5 mM H_2SO_4 at 55 ° C. The NAD/NADH Quantitation Kit for NADH detection, the NADPH Quantitation Kit for sigam for NADPH detection.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LL conceived of the study, performed the data analysis, and coordinated the manuscript draft and revision. SY, TY, XF executed the experimental work and data analysis. TY, ZX, ZR, MX, sl helped to revise and proofread, coordinated the manuscript.

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Abbreviations

2,3-BDO:2,3-butanediol; PPP:Pentose Phosphate Pathway; EMP:Glycolysis Pathway; GDH:glucose dehydrogenase; TDH:NADPH-dependent 2.3-BDO dehydrogenase; ALS:cetolactate synthase; ALDC:acetolactate decarboxylase; GRAS:generally regarded as safe; LdhA:lactate dehydrogenase.

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Tables

Table 1 Strains used in this study

Strains	Relevant characteristic or sequence	Source
BM0	*B.subtilis168*	Lab stock
DH5a	*E. coli*	Lab stock
BM1	*B. subtilis 168*pMA5-P_{bdhA}-*gdh*	This study
BM2	*B. subtilis 168*pMA5-P_{pac}-*gdh*	This study
BM3	*B. subtilis 168*pMA5-P_{spac}-*gdh*	This study
BM4	*B. subtilis 168*pMA5-P_{HpaII}-*gdh*	This study
BM5	*B. subtilis 168*pMA5-*TDH*	This study
BM6	*B. subtilis 168*pMA5-P_{HpaII}-*TDHR1*	This study
BM7	*B. subtilis 168*pMA5-P_{HpaII}-*TDHR2*	This study
BM8	*B. subtilis 168*pMA5-P_{HpaII}-*TDHR3*	This study
BM9	*B. subtilis 168*pMA5-P_{HpaII}-*TDHR4*	This study
BM10	*B. subtilis 168*pMA5-P_{HpaII}-*TDHR5*	This study
BM11	*B. subtilis 168* pMA5-P_{HpaII}-*TDHR5*-P_{bdhA}-*gdh*	This study
BM12	*B. subtilis 168* pMA5-P_{HpaII}-*TDHR5*-P_{pac}-*gdh*	This study
BM13	*B. subtilis 168* pMA5-P_{HpaII}-*TDHR5*-P_{spac}-*gdh*	This study
BM14	*B. subtilis 168* pMA5-P_{HpaII}-*TDHR5*-P_{HpaII}-*gdh*	This study
BM15	*B. subtilis 168ΔldhA* P_{srfA}-*alsr*: pMA5-P_{HpaII}-*TDHR5*-P_{pac}-*gdh*	This study

Table 2 plasmids and primers used in this study

Plasmid or Primer	Relevant characteristic	Source
Plasmid		
p7Z6	zeo^r, Amp^r containing zeo and cre, lox gene	Lab stock
pDG148	Kan^r	Lab stock
pMA5	*E. coli–B. subtilis* shuttle vector (in E. coli, Ap ^{r} ; in B. subtilis, Kan ^{r})	Lab stock
pMA5-P_{bdhA}-*gdh*	*gdh* under P_{bdhA} promote	This study
pMA5-P_{pac}-*gdh*	*gdh* under P_{pac} promote	This study
pMA5-P_{spac}-*gdh*	*gdh* under P_{spac} promote	This study
pMA5-P_{HpaII}-*gdh*	*gdh* under P_{HpaII} promote	This study
pMA5-P_{HpaII}-*TDH*	*TDH* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR1*	*TDHR1* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR2*	*TDHR2* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR3*	*TDHR3* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR4*	*TDHR4* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR5*	*TDHR5* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR5*-P_{bdhA}-*gdh*	*TDHR5* and *gdh* under P_{bdhA} promoter	This study
pMA5-P_{HpaII}-*TDHR5*-P_{pac}-*gdh*	*TDHR5* and *gdh* under P_{pac} promoter	This study
pMA5-P_{HpaII}-*TDHR5*-P_{HpaII}-*gdh*	*TDHR5* and *gdh* under P_{HpaII} promoter	This study
pMA5-P_{HpaII}-*TDHR5*-P_{Spac}-*gdh*	*TDHR5* and *gdh* under P_{spac} promoter	This study
Primer		
P1	GCATCGCGCGCGGGGAATTCCGCACAATCCCAATGTCCAAGT	
P2	CGACTTTTCCTTTTAAATCCGGATACATGGATTACCACTCCTATAACTTTTGATGT	
P3	GCATCGCGCGCGGGGAATTCGGTGGAAACGAGGTCATCATTTCC	
P4	CTTTTAAATCCGGATACATCAAAATCGTCTCCCTCCGT	
P5	GCATCGCGCGCGGGGAATTCGAATTCTACACAGCCCAGTCCAG	
P6	CTTTTAAATCCGGATACATGAATTCTAGATACACCTCCTTAAGCTT	
P7	GGCATCGCGCGCGGGGAATTCCTGAGTCTGGCTTTCGGTAAGC	
P8	TTTTCCTTTTAAATCCGGATACATTAAATCGCTCCTTTTTAGGTGGCAC	
P9	ATGTATCCGGATTTAAAAGGAAAAGTCG	
P10	TCGAGCTCTCCCGGGAATTCTTAACCGCGGCCTGCC	
P11	GCTCGACTCTAGAGGATCCTTAAAGAAGAGACTTGTCTGG	
P12	AAAGGAGCGATTTACATATGATGAAGGCCGTTCTTTGGTA	

P13	GCTCGACTCTAGAGGATCCTTAAAATTTAAGAAGAGACTTGTCTGGCGT
P14	GCTCGACTCTAGAGGATCCTTATTTAAATTTAAGAAGAGACTTGTCTGGCGT
P15	GCTCGACTCTAGAGGATCCTTAAAATTTAAATTTAAGAAGAGACTTGTCTGGCGT
P16	GCTCGACTCTAGAGGATCCTTATTTTTTAAATTTAAGAAGAGACTTGTCTGGCGT
P17	GCTCGACTCTAGAGGATCCTTATTTTTTAAGAAGAGACTTGTCTGGCGT
P18	ACCATGATTACGAATTCGAGCTC
P19	ACGTTGTAAAACGACGGCC
P20	TGGCTGGACAGCCTGAGG
P21	GAGCTCGAATTCGTAATCATGGTTAATCATCCTTCCAGGGTATGTTTCTC
P22	GGCCGTCGTTTTACAACGTCCGCAACTTTAGAGTAAAGGGCT
P23	CAGCCCGCCTTCTTGGAA
P24	GGTACCTCTAGAAGAAGCTTATCGACAAAAATGTCATGAAAGAATCGT
P25	AGATGGCGAAGCTCCATATTGTCATACCTCCCCTAATCTTTATAAGC
P26	ATGGAGCTTCGCCATCTTCAA
P27	TCGAGCTCTCCCGGGAATTCTCATGTACCTGCATCACTCTC
P28	CTGGGAGAAAAATGCCGAGAT
P29	GGAAACTCGTGGCGATAAGC
P30	TCCACGCCGTAAACGATGA
P31	TTCCTTTGAGTTTCAGTCTTGCG

Table 3 Metabolic characterizations of B. subtilis strains cultivated in fermentation medium supplemented with 100 g/L glucose

Strains	Time(h)	OD_{600}	2,3-BDO (g/L)	AC (g/L)	Lactate (g/L)	Ethanol (g/L)	Intracellular NADH (μmol/L/OD_{600})	Intracellular NADPH (μmol/L/OD_{600})
BM0	72±2	15.20±0.13	34.60±0.29	14.75±0.15	5.46±0.19	0.93±0.05	1.90±0.13	1.49±0.15
BM1	78±1.5	14.32±0.34	29.36±0.39	17.35±0.37	4.63±0.31	0.63±0.12	1.80±0.11	1.69±0.13
BM2	80±3	14.25±0.56	28.25±0.50	18.06±0.45	4.49±0.39	0.61±0.13	1.74±0.09	1.72±0.17
BM3	84±2	14.23±0.23	27.83±0.83	19.54±0.54	4.35±0.36	0.59±0.23	1.69±0.07	1.78±0.14
BM4	84±1.5	14.03±0.19	26.90±0.75	20.31±0.38	4.20±0.26	0.58±0.28	1.62±0.01	1.83±0.11
BM11	72±2	14.15±0.25	38.13±0.19	12.03±0.24	4.83±0.30	0.86±0.03	1.86±0.02	1.51±0.09
BM12	72±2	14.38±0.16	39.45±0.23	11.41±0.16	4.76±0.35	0.71±0.07	1.83±0.05	1.54±0.12
BM13	73±1.5	14.25±0.45	37.45±0.25	12.35±0.26	4.68±0.19	0.69±0.09	1.79±0.07	1.58±0.16
BM14	73±1.5	14.24±0.36	36.03±0.13	12.45±0.17	4.58±0.24	0.60±0.08	1.75±0.05	1.64±0.10

The intracellular NADH and NADPH of BM1、BM2、BM3、BM4 were extracted at 72 h、The intracellular NADH and NADPH of other strains were extracted at 60 h.

Figures

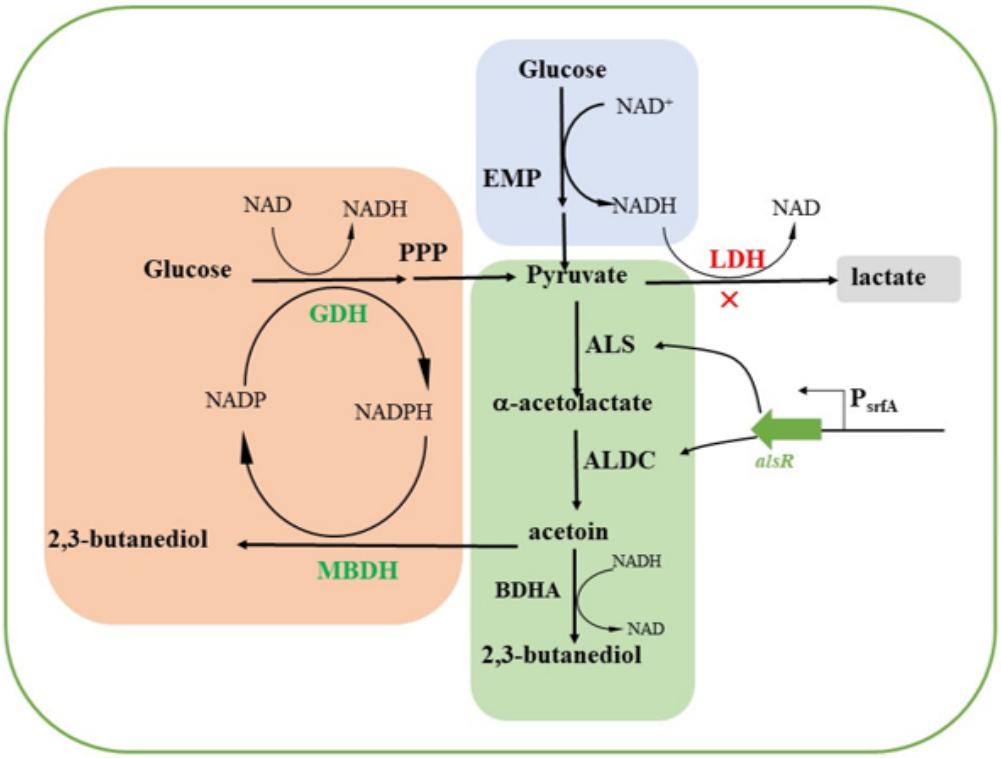

Figure 1

The 2,3-BDO biosynthetic pathway in B. subtilis GDH, glucose dehydrogenase; ALS, acetolactate synthase; ALDC, acetolactate decarboxylase; LDH, lactate dehydrogenase; AlsR, transcriptional regulator; TDH, NADPH-dependent 2, 3-

butanediol dehydrogenase.

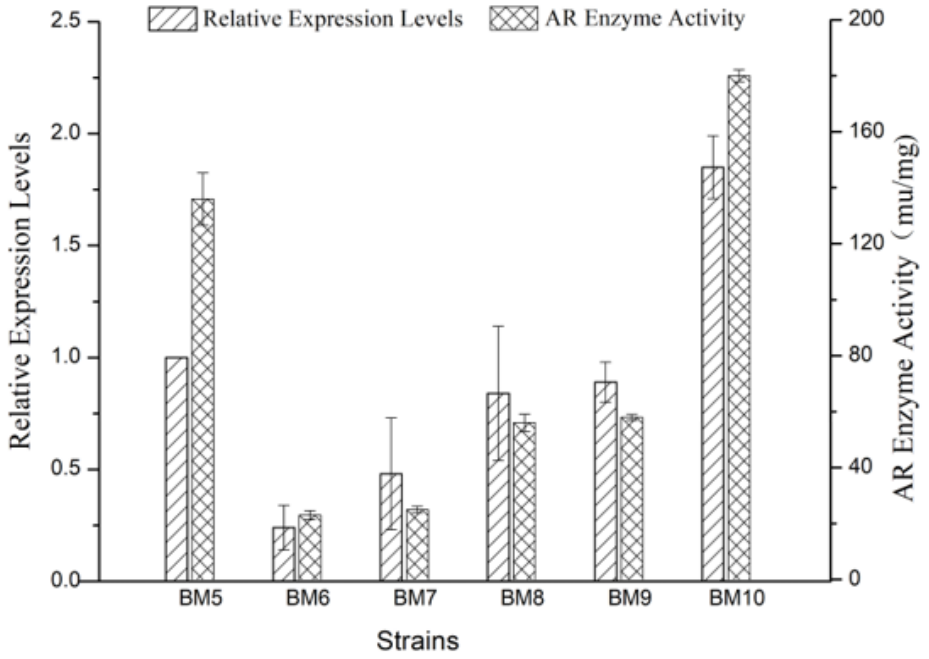

Figure 2

Transcriptional levels and activity of TDH in the recombinant strains. BM5, the control strain; BM6, strain with TDHR1; BM7, strain with TDHR2; BM8, strain with TDHR3; BM9, strain with TDHR4; BM10, strain with TDHR5; the 16S rRNA gene was used as the internal control gene to normalize the results. Error bars: Standard deviation (SD) (n =3).

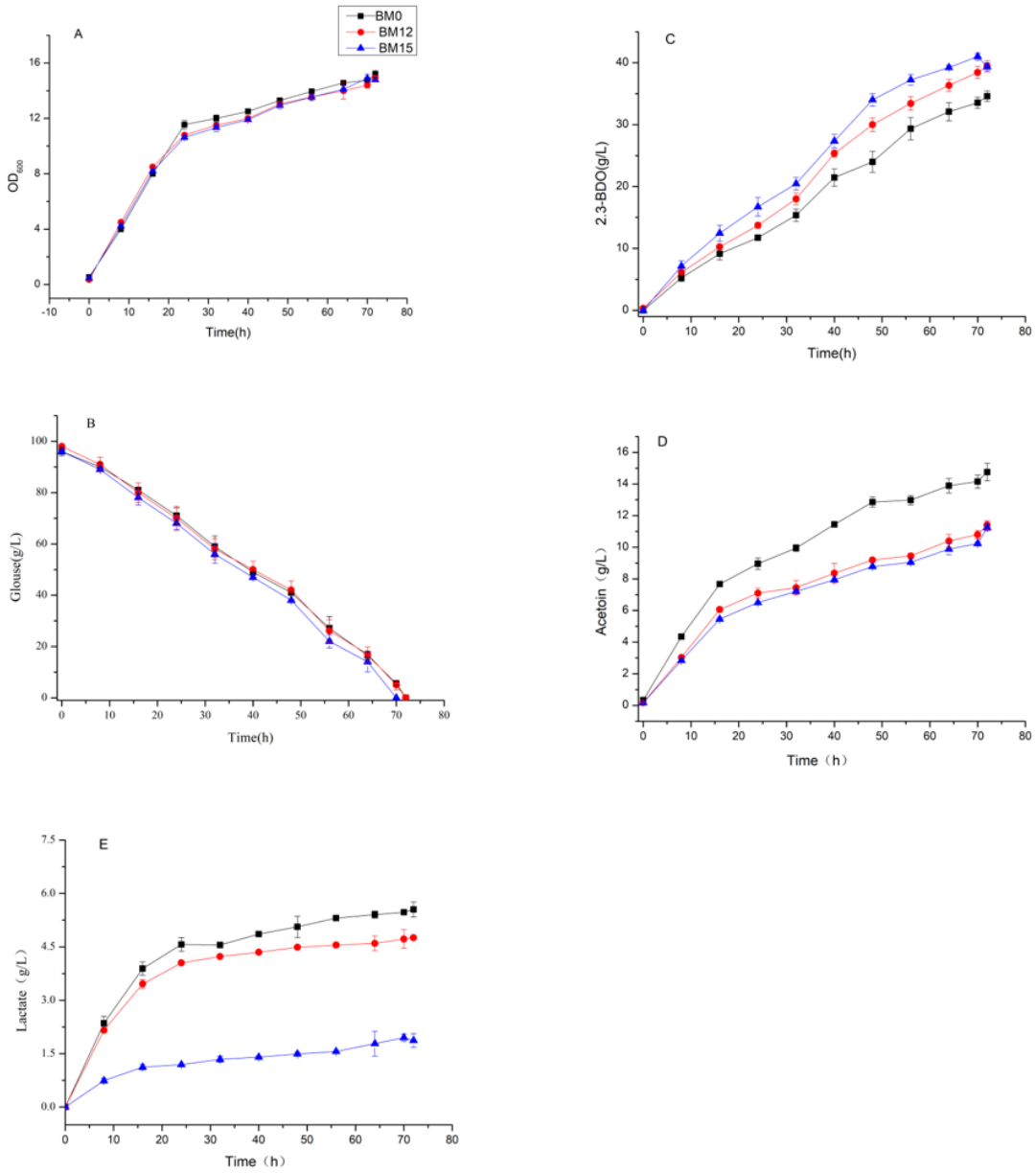

Figure 3

Time profiles of 2,3-BD fermentation with various strains (A cell growth; B glucose consumption; C 2,3-BD production; D acetoin formation; E lactate production). Fermentation was carried out at 37 °C in 50 mL fermentation medium. BM0 strain, B. subtilis 168 BM12 strain, B. subtilis 168 with plasmid pMA5-PHpaII-TDHR5-Ppac-gdh; BM15 strain, B. subtilis 168ΔldhA PsrfA-alsr: plasmid pMA5-PHpaII-TDHR5-Ppac-gdh;) Error bars: Standard deviation (SD) (n =3).