

Metabolic engineering of *Bacillus subtilis* to enhance the production of tetramethylpyrazine

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Abstract

Objective *Bacillus subtilis* BS2, which can produce tetramethylpyrazine (TTMP) from glucose, was engineered by knockout of the 2,3-butanediol (2,3-BD) dehydrogenase gene (*bdhA*) and then regulated through the addition of 2,3-BD to enhance the TTMP yield.

Results The *bdhA* of *B. subtilis* BS2 was disrupted to construct a TTMP-producing strain termed BSA. In microaerobic flask fermentation, the BSA strain produced 27.8 g TTMP/l. This was 6 g/l higher than that produced by the initial strain. Compared with that in BS2, the maximum yield of acetoin, which is a TTMP precursor, also increased from 11.3 to 16.4 g/l in BSA. The TTMP production by BS2 was enhanced by 2,3-BD supplemented to the fermentation medium. The maximum TTMP and acetoin yields were improved from 21.8 to 29.7 g/l and from 11.3 to 15.4 g/l, respectively, as the 2,3-BD concentration increased from 0 to 3 g/l. Conversely, the yields did

not increase when the 2,3-BD concentration in the matrix was ≥ 4 g/l.

Conclusions This study provides valuable information to enhance the TTMP productivity of mutagenic strains through gene manipulation and fermentation optimization.

Keywords Acetoin · *Bacillus subtilis* · 2,3-Butanediol · 2,3-Butanediol dehydrogenase · Metabolic engineering · Tetramethylpyrazine

Introduction

Tetramethylpyrazine (TTMP) is a heterocyclic, nitrogen-containing compound responsible for different food flavors, such as nutty and roasty (Muller and Rappert 2010; Xiao et al. 2006). It is therefore used as a food additive to enhance flavor. TTMP is also an important aromatic compound in some Chinese liquors (Fan et al. 2007). Moreover, TTMP is a key component of *Ligusticum chuanxiong* Hort to cure cardiovascular and cerebrovascular diseases (Ai-ping et al. 1986). Pharmacological studies have confirmed that TTMP can dilate blood vessels, increase coronary blood flow, and inhibit platelet aggregation. TTMP serves as a platform compound commonly applied in many industries other than the food and beverage industry.

TTMP can be produced through chemical or biological synthesis (Amrani-Hemaimi et al. 1995;

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Zhu et al. 2010). Kosuge et al. (1962) provided the first evidence that microorganisms can synthesize pyrazines; showing that TTMP can be produced by *Bacillus subtilis*. TTMP in liquor mainly comes from microbial metabolism and not from the Maillard reaction (Yan et al. 2011). A newly proposed theory is related to the TTMP synthesis in Chinese liquor through solid-state fermentation with enzyme/thermal dynamic coupling catalysis to synthesize TTMP (Jianfeng and Yan 2014). *Bacillus* sp. can produce a high TTMP yield via the precursor acetoin by using an endogenous precursor screening strategy (Zhu et al. 2010). Compared with chemical synthetic and enzyme conversion methods, the microbial fermentation route provides several advantages, such as environmentally friendly and cost-effective processes.

Although *B. subtilis* has been engineered to produce acetoin it has yet to be developed to produce TTMP through gene modification. Our current interest focuses on the genetic alterations of carbon flux into the acetoin biosynthesis pathway by blocking the degradation and competing pathways; thus, acetoin can accumulate to enhance the TTMP yield. The final titer and TTMP yield from glucose of the engineered strain proved higher than those of the parent strain. This study also developed a novel method with 2,3-butanediol (2,3-BD) supplementation during fermentation to improve TTMP production at a low cost.

Materials and methods

Strains, plasmids, primers and medium

The strains, plasmids, and their relevant genotypes used in this study are listed in Supplementary Table 1. The PCR primers used in this study are listed in Supplementary Table 2. DNA was manipulated by using standard protocols. *Escherichia coli* was grown at 37 °C in lysogeny broth (LB) (composed of 10 g NaCl/l, 10 g tryptone/l, and 5 g yeast extract/l) supplemented with 100 µg ampicillin/ml to select positive *E. coli* transformants. In tetramethylpyrazine (TTMP) and acetoin fermentation, 70 g glucose/l was added to LB, and 5 ml supplement (1 mg glucose/ml) was added every 12 h. Furthermore, LB plates were supplemented with 100 µg filter-sterilized kanamycin/ml to select *B. subtilis* transformants harboring the *KanMX* gene. The solid media contained 20 g agar/l.

Construction of *bdhA* knockout mutants

The oligonucleotides used to construct plasmids are listed in Supplementary Table 2. A series of plasmids was constructed (Fig. 1) to establish a genome integration cassette. First, a 423 bp promoter region of 2,3-BD dehydrogenase gene (*bdhA*) was amplified from the genomic DNA of *B. subtilis* BS2 by using the primer pair *bdhA* -AU/*bdhA* -AD. The PCR product was cut with *EcoRI/KpnI*, and then inserted into pUC19 (Invitrogen, China) cut with *EcoRI/KpnI* to yield pUC-bSA. Likewise, a 511 bp terminator region of *bdhA* was amplified through PCR from the genomic DNA of *B. subtilis* BS2 by using the primer pair *bdhA* -BU/*bdhA* -BD, cut with *BamHI/HindIII*, and inserted into pUC-bSA cut with *BamHI/HindIII* to yield the pUC-bSAB plasmid. Next, the kanamycin (Kan) resistance cassette was amplified from the pET28a plasmid by using the primer pair *kan-up/kan-down*. The PCR product with a size of approximately 1249 bp was purified, digested with *BamHI/KpnI* and inserted into pUC-bSAB cut with the same enzyme pair to yield pUC-bSABK.

TTMP production of the wild-type and mutant strains

A single colony of *B. subtilis* BS2 cells was transferred into 5 ml LB containing the corresponding antibiotic as required. Cultivations were performed at 37 °C. After 12 h shaking at 200 g, a 2 % (v/v) inoculum was added to 50 ml LB with 10 g glucose/l in a 250 ml shake-flask and cultivated for 12 h. This culture was inoculated at 4 % (v/v) into 200 ml LB with 70 g glucose/l in a 500 ml flask and shaken at 200 g for 8 days. The initial OD₆₀₀ was ~0.05. Five ml of supplement (1 mg glucose/ml) was added every 12 h. pH was controlled at 7.5 by adding 10 M NaOH.

2,3-BD addition to the TTMP production

At the beginning of fermentation, 2,3-BD was added to the medium at initial concentrations of 1, 2, 3, 4 and 5 g/l.

Analytical methods

The biomass of the fermentation broth was determined from the OD₆₀₀ value. Glucose concentration of the fermentation broth was determined by using a SBA-40C biosensor.

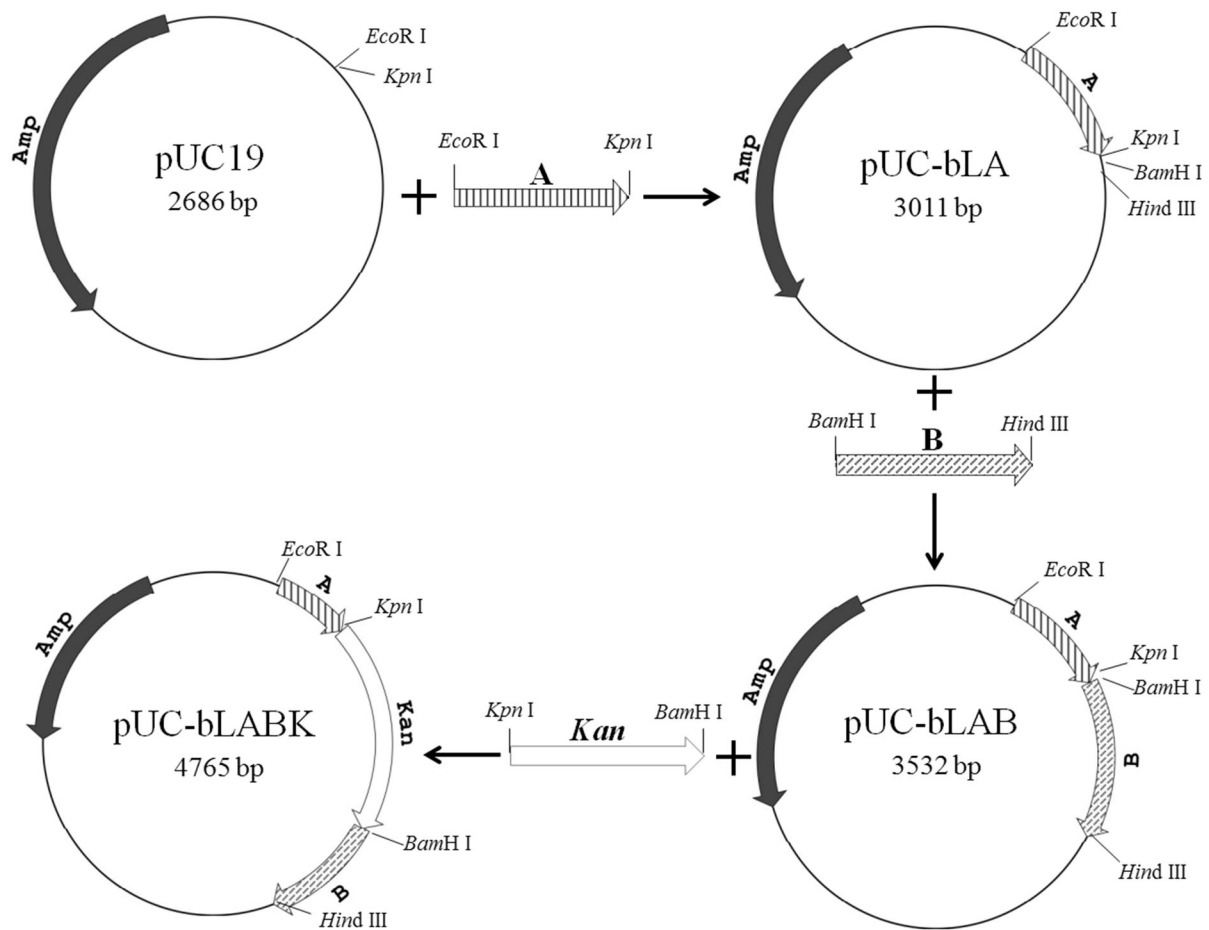


Fig. 1 Construction of pUC-bSABK

The TTMP concentration was determined through headspace solid-phase micro-extraction and GC-nitrogen, as reported previously. The concentrations of acetoin and 2,3-BD were determined by GC (Gao et al. 2014; Shi et al. 2014).

Results and discussion

Characterization of the *bdhA*-disrupted transformants

The 2,3-butanediol (2,3-BD) synthesis pathway was blocked by disrupting the 2,3-BD dehydrogenase gene (*bdhA*), and acetoin, which is the tetramethylpyrazine (TTMP) precursor (Fig. 2), can accumulate to increase the yield of TTMP. Compared with the acetoin yield of the initial strain (*B. subtilis* BS2), the maximum

acetoin yield increased by 45 % (w/w) from 11.3 to 16.4 g/l; the maximum TTMP yield also increased by 27.5 % (w/w) from 21.8 to 27.8 g/l in the *bdhA*-disrupted mutant strain (*B. subtilis* BSA). By contrast, 2,3-BD production decreased by 72.4 % (w/w) (Fig. 3 a).

Compared with those in BS2, the TTMP and acetoin production in the BSA strain increased by 27.5 % (w/w) and 45 % (w/w), respectively, when the strains were cultivated for 168 h and 144 h, respectively (Fig. 3a); this increase was mainly due to (i) the abolished 2,3-BD production in the early stationary phase and (ii) the accumulated precursor acetoin in the early stationary phase. BS2 accumulated more than 9 g 2,3-BD/l when cultivated for 72 h, and the BSA strain accumulated <3 g 2,3-BD/l when cultivated for 72 h in the medium with 1 g residual glucose/l. The amount of 2,3-BD decreased in the stationary phase

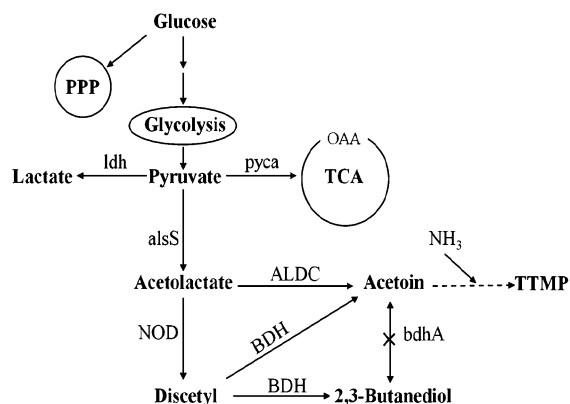


Fig. 2 Tetramethylpyrazine (TTMP) biosynthetic pathway and other overflow metabolism pathways in *Bacillus subtilis* BS2. *AlsS*, *ALDC*, and *bdhA* encode acetolactate synthase, acetolactate decarboxylase, and 2,3-butanediol (2,3-BD) dehydrogenase, respectively. *PPP* Pentose phosphate pathway; *TCA* tricarboxylic acid cycle; *NOD* oxidative decarboxylation of non-enzymatic catalysis. Disrupted pathway steps are indicated by arrow breaks

(Fig. 3a). This result is consistent with that described in a previous study, which suggested the degradation pathway of 2,3-BD via acetoin as an intermediate (Thanh et al. 2010).

The amount of acetoin accumulated by BS2 decreased at 120 h (Fig. 3a) because of acetoin degradation. Acetoin is mainly used as a precursor of TTMP or as a carbon source for growth (Huang et al. 1999). A high acetoin concentration accumulates in the BSA strain; as a result, a high TTMP concentration is produced; nevertheless, acetoin is

metabolized more slowly by BSA than by BS2 (Silbersack et al. 2006).

Effect of 2,3-BD supplemented to the medium

The effect of 2,3-BD on the TTMP and acetoin production by BS2 was investigated by adding 1, 2, 3, 4, and 5 g/l of 2,3-BD to the medium. TTMP production was affected by 2,3-BD supplemented to the fermentation medium. The TTMP and acetoin production improved when 1–3 g/l of 2,3-BD was added to the culture medium; by contrast, this supplementation slightly influenced cell growth (Table 1), residual glucose concentration, and 2,3-BD production by BS2 (Fig. 4). TTMP increased as the 2,3-BD dosage increased, but no increase was observed when the supplemented 2,3-BD in matrix was ≥ 4 g/l (Table 1). Compared with those of BS2 (no dosage), the maximum TTMP and acetoin yields increased by 36.2 % (w/w) and 36.3 % (w/w) in BS2R3 (BS2 with 3 g 2,3-BD/l), respectively; this increase was observed when the strains were cultivated for 144 h and 168 h, respectively (Fig. 3b). In general, 3 g 2,3-BD/l gives the maximum production.

The TTMP and acetoin yields improved from 21.8 to 29.7 g/l and from 11.3 to 15.4 g/l, respectively, as the 2,3-BD dosage increased from 0 to 3 g/l. Thus, 2,3-BD can dose-dependently promote the TTMP and acetoin yields. The maximum TTMP and acetoin yields were reached at 3 g 2,3-BD/l in the culture medium. These results suggested that the methodology is a cost-effective option for the TTMP and

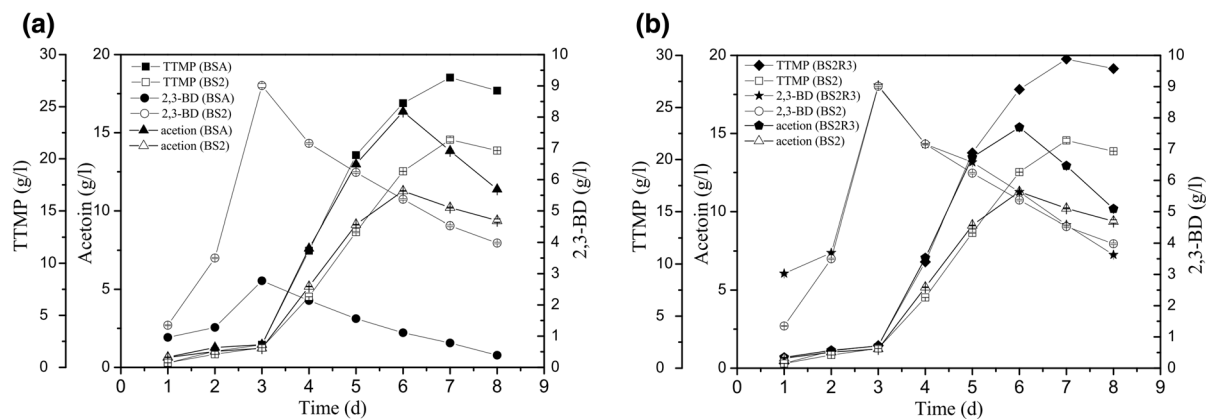


Fig. 3 TTMP, acetoin, and 2,3-BD concentrations of BS2, BSA, and 2,3-BD-regulated BS2 (BS2R3) cultivated with 200 ml of LB in 500 ml flask shaken at 200 g and 37 °C for

8 days. **a** Effects of *bdhA* disruption on TTMP, acetoin and 2,3-BD production by *B. subtilis* BSA. **b** Effects of 2,3-BD addition on TTMP, acetoin and 2,3-BD production by *B. subtilis* BS2

Table 1 Effect of 2,3-BD addition on TTMP production by *Bacillus subtilis* BS2

Cultivation conditions				Time ^a (h)	Yield of acetoin (g/l)	Time ^b (h)	Yield of TTMP (g/l)	OD ₆₀₀ ^{max}
Initial glucose concentration (g/l)	pH	T (°C)	2,3-BD addition (g/l)					
70	7.5	37	0	144	11.3	168	21.8	12.2
			1	144	13.1	168	22.2	13.7
			2	144	13.4	168	24.3	14.8
			3	144	15.4	168	29.7	17.7
			4	144	13.2	168	25.7	15.3
			5	144	10.4	168	18.7	11.2

T Temperature

^a Time in hours from inoculation to the maximal Acetoin concentration arrived

^b Time in hours from inoculation to the maximal TTMP concentration arrived

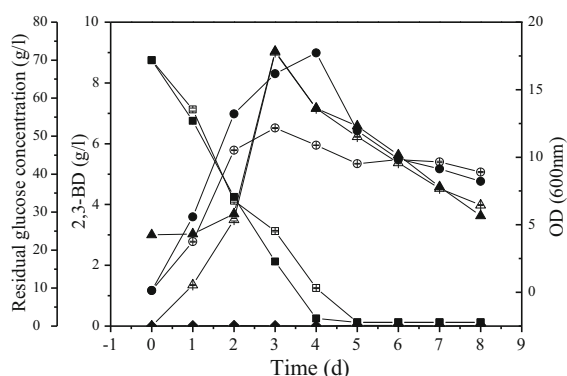


Fig. 4 Effect of 2,3-BD addition on cell growth (filled circle), 2,3-BD production (filled triangle) and residual glucose concentration (filled square) by *B. subtilis* BS2. Product profiles of BS2 (open) and BS2R3 (filled symbols) are shown

acetoin production. Nevertheless, the mechanism by which acetoin synthesis is improved by 2,3-BD supplementation is poorly understood. AR/BDH likely catalyzes the conversion between AC and BD (Juni and Heym 1956); the initial 2,3-BD causes feedback regulation to catalyze the conversion from BD to AC; as a result, the concentration of AC increases and the concentration of BD decreases (Fig. 3b). After 2,3-BD was added, BS2 accumulates a high concentration of acetoin and produces a high amount of TTMP (Fig. 3b); 2,3-BD is slightly conducive to cell growth (Fig. 4). Enzyme activities reach the peak in the presence of high carbohydrate concentrations and rapidly decrease after carbon sources are depleted (Skory 2000); thus, the initial suppression on AR/BDH

by 2,3-BD causes a very low activity and impedes the synthesis of 2,3-BD when sugars are almost completely exhausted at the end of fermentation. Furthermore, 2,3-BD can be used as a carbon source to generate acetoin when the initial concentration of the carbon source in the medium is low (Zhang et al. 2011). When 2,3-BD is added at an inhibitory concentration, cell growth is adversely affected and product synthesis is inhibited (Table 1). However, the inhibitory mechanism of 2,3-BD remain unclear. This mechanism is a possible result of enzymes activation or inhibition by 2,3-BD during synthesis; this mechanism is also a possible consequence of the transfer induced by different enzymes. Therefore, the inhibitory mechanism of 2,3-BD should be further investigated.

Conclusion

An engineered BSA strain giving a high yield of tetramethylpyrazine (TTMP) was constructed. The alteration of carbon flux into the acetoin biosynthetic pathway was demonstrated as a direct and effective strategy to enhance the TTMP yield. Acetoin catabolic and competing pathways were blocked by disrupting the 2,3-butanediol (2,3-BD) dehydrogenase gene (*bdhA*), which abolished 2,3-BD production and accumulated the precursor acetoin accumulation in the early stationary phase. In flask fermentation, the TTMP and acetoin yields respectively increased by 27.5 % (w/w) and 45 % (w/w) in BSA compared with

BS2. Different concentrations of 2,3-BD were supplemented to the fermentation medium to improve the TTMP and acetoin production by BS2. The supplementation of 2,3-BD to the substrate is a novel and efficient method to improve the TTMP and acetoin production. Our results showed that the TTMP and acetoin yields respectively increased by 36.2 % (w/w) and 36.3 % (w/w) acetoin in BSA compared with BS2. Nevertheless, the proposed mechanism should be further investigated.

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Supporting information Supplementary Table 1—Bacterial strains and plasmids used.

Supplementary Table 2—Primers used.

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