World J Microbiol Biotechnol (2016)32:46
DOI 10.1007/s11274-015-1992-1

ORIGINAL PAPER

Enhanced production of tetramethylpyrazine in *Bacillus licheniformis* BL1 by *bdhA* disruption and 2,3-butanediol supplementation

Wu Meng[1,2] · Dongguang Xiao[2] · Ruiming Wang[1]

Received: 31 August 2015 / Accepted: 7 December 2015

Abstract The 2,3-butanediol (2,3-BD) dehydrogenase gene (*bdhA*) of *Bacillus licheniformis* BL1 was disrupted to construct the tetramethylpyrazine (TMP)-producing BLA strain. During microaerobic fermentation, the *bdhA*-disrupted BLA strain produced 46.98 g TMP/l, and this yield was 23.99 % higher than that produced by the parent BL1 strain. In addition, the yield of acetoin, which is a TMP precursor, also increased by 28.98 % in BLA. The TMP production by BL1 was enhanced by supplementing the fermentation medium with 2,3-BD. The yield of TMP improved from 37.89 to 44.77 g/l as the concentration of 2,3-BD increased from 0 to 2 g/l. The maximum TMP and acetoin yields increased by 18.16 and 17.87 %, respectively with the increase in 2,3-BD concentration from 0 to 2 g/l. However, no increase was observed when the concentration of 2,3-BD in the matrix was ≥3 g/l. This study provides a valuable strategy to enhance TMP and acetoin productivity of mutagenic strains by gene manipulation and optimizing fermentation conditions.

Keywords *Bacillus licheniformis* · *bdhA* gene · Metabolic engineering · TMP · Yield

✉ Ruiming Wang
ruiming3k@163.com

Dongguang Xiao
xiaodongguang123@163.com

[1] Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology, Jinan 250353, Shandong, China

[2] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, Tianjin University of Science and Technology, TEDA, Tianjin 300457, China

Introduction

Tetramethylpyrazine (TMP) is a heterocyclic, nitrogen-containing compound with a roasted nutty flavor (Muller and Rappert 2010; Xiao et al. 2006), found in a wide variety of foods as a flavoring agent. It is an important aromatic compound in some Chinese liquors (Fan et al. 2007). It is also the key component in *Ligusticum chuanxiong* Hort that is used to cure cardiovascular and cerebrovascular diseases (Ai-ping et al. 1986). Pharmacological studies have confirmed that TMP can dilate blood vessels, increase coronary blood flow, and inhibit platelet aggregation. In addition, this compound is used in the food industry as flavour additives (Lancker et al. 2010, 2012). Apart from its wide use in the food and beverage industry, TMP also serves as a standard compound that is widely utilized in many other industries (Hao et al. 2013).

Production of TMP can be achieved by chemical or biological synthesis (Amrani-Hemaimi et al. 1995; Zhu et al. 2010). The alanine and glycine are two major substrates to form pyrazines in the Maillard Model Systems, and previous research indicated that alanine and glycine not only provide the nitrogen source but also contribute to the alkyl side chain of some alkylpyrazines (Amrani-Hemaimi et al. 1995; Lancker et al. 2011). The first evidence that microorganisms are able to synthesize pyrazines was provided by Kosuge et al. (1962), who showed that TMP could be produced by *Bacillus subtilis*. Another study proved that TMP in Chinese white liquor is mainly generated from microbial metabolism and not by the Maillard reaction (Yan et al. 2011). A new theory has been proposed and demonstrated in which TMP is synthesized in Chinese liquor by solid-stage fermentation with enzyme/thermal dynamic coupling catalysis mechanisms (Jianfeng and Yan 2014). *Bacillus* sp. can produce a high TMP yield from the

Published online: 12 February 2016

precursor acetoin by using an endogenous precursor screening strategy (Zhu et al. 2010). Compared with synthetic chemical and enzyme conversion methods, the microbial fermentation route is advantageous in being more environmentally friendly and cost-effective (Zhang et al. 2011).

To our knowledge, *Bacillus licheniformis* has yet to be utilized to produce TMP through gene modification. Our current interest focuses on the genetic alterations of carbon flux in the acetoin biosynthesis pathway by blocking the degradation and competing pathways. The acetoin then accumulates to enhance the TMP yield. The final titer and the TMP yield from glucose of the engineered strain is higher than those of the parent strain. We also tested a novel method of supplementing 2,3-butanediol (2,3-BD) during the fermentation process to effectively improve TMP production at a low cost.

Materials and methods

Strains, plasmids, primers and medium

Table 1 lists all the strains and plasmids as well as their relevant genotypes used in this study. PCR primers used in this study are listed in the 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/l NaCl, 10 g/l tryptone, and 5 g/l yeast extract) supplemented with ampicillin (100 μg/ml) to select for positive *E. coli* transformants. In tetramethylpyrazine (TMP) and acetoin fermentation, 70 g/l glucose was supplemented in the LB broth, and 5 ml of the supplement (1 mg/ml glucose) was added every 12 h. In addition, LB plates were supplemented with 100 μg/ml filter-sterilized kanamycin antibiotic to select for *B. licheniformis* transformants harboring the *KanMX* gene. The solid media contained 20 g/l agar.

Construction of *bdhA* knock-out mutants

The oligonucleotides used to construct the plasmids are listed in Table 2. A series of plasmids were constructed (Fig. 1) to establish a genome integration cassette. First, a 342 bp promoter region of *bdhA* was amplified from the genomic DNA of *B. licheniformis* BL1 by using the primer pair *bdhA*-AU/*bdhA*-AD. The PCR product was cut with *Eco*RI/*Kpn*I, and then inserted into pUC19 (Invitrogen, Thermo Fisher Scientific Inc., Shanghai, China) that is also cut with *Eco*RI/*Kpn*I, to yield pUC-bLA. Following the generation of pUC-bLA, a 538 bp terminator region of *bdhA* was amplified through PCR from the genomic DNA of *B. licheniformis* BL1 by using the primer pair *bdhA*-BU/*bdhA*-BD and cut with *Bam*HI/*Hin*dIII. The cleaved terminator region is then inserted into pUC-bLA that is also cut with *Bam*HI/*Hin*dIII to yield the pUC-bLAB plasmid. The kanamycin (Kan) resistance cassette was then 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 *Bam*HI/*Kpn*I, and inserted into pUC-bLAB cut with the same enzyme pair to yield pUC-bLABK.

Production of TMP by wild-type and mutant strains

The single colony of *B. licheniformis* BL1 cells was transferred into 5 ml of LB medium containing the corresponding antibiotic as required. All cultivations were performed at 37 °C. After 12 h of shaking at 200 rpm, 2 % (v/v) inoculum was added to 50 ml LB with 10 g/l glucose in a 250 ml flask and cultivated for 12 h. This culture was inoculated with 4 % (v/v) inoculum at an initial optical density (OD_{600}) of 0.05 under the following culture conditions for fermentation: 200 ml of LB with 70 g/l glucose in a 500 ml flask was shaken at 200 rpm for 8 days, and 5 ml of supplement (1 mg/ml glucose) was added every 12 h. pH was controlled at 7.5 by adding 10 M NaOH.

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	Host strain in gene cloning/general cloning and storage of plasmids	Laboratory stock
B. licheniformis BL1	For production of TMP	Laboratory stock
B. licheniformis BLA	The mutant derived from *B. licheniformis* BL1	Laboratory stock
Plasmids		This study
pUC19	For construction of the gene knockout vector	Laboratory stock
pUC-bLABK	For *bdhA* knockout	This study
pET28a	Source of *KanMX*	Laboratory stock

Table 2 Primers used in this study

Primer	Sequence (5′ → 3′)	Digestion site
bdhA-AU	CCGGAATTCGCATTGTGCGGGTTC	*Eco*R I
bdhA-AD	GGGGTACCTCCTCTGCCAATCGT	*Kpn* I
bdhA-BU	CGGGATCCTATCCGAGCCGGAAG	*Bam*H I
bdhA-BD	CCCAAGCTTTTGAGGGCGAAGTTGC	*Hind* III
kan-up	CGGGATCCGCTCTGCTGAAGCCAGTTACCTT	*Bam*H I
kan-down	GGGGTACC ATTTTGCCGATTTCGGCCTATTG	*Kpn* I

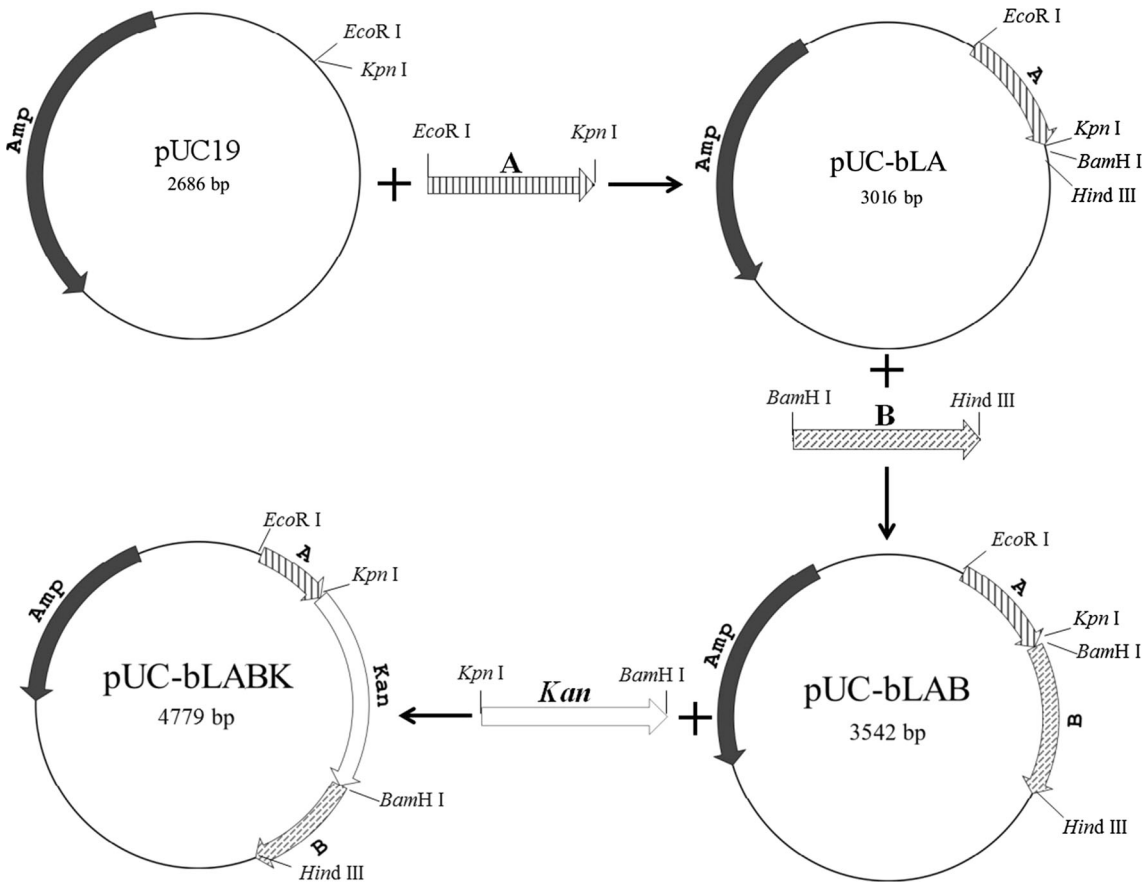

Fig. 1 Construction of plasmid pUC-bLABK

Analytical methods

The biomass of the fermentation broth was determined at different times and at OD of 600 nm in a spectrophotometer (UV-722, Shanghai Xinmao Instrument Company Limited, China). The glucose concentration of the fermentation broth was determined at different times by using an enzymatic membrane biosensor (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China) with a glucose oxidase-immobilized membrane.

The TMP concentration was determined through headspace solid-phase microextraction and gas chromatography-nitrogen, as reported previously (Sabik et al. 2012; Lancker et al. 2010). The concentrations of acetoin and 2,3-BD were determined through gas chromatography (Gao et al. 2014; Shi et al. 2014).

Results

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*). Acetoin, a tetramethylpyrazine (TMP) precursor

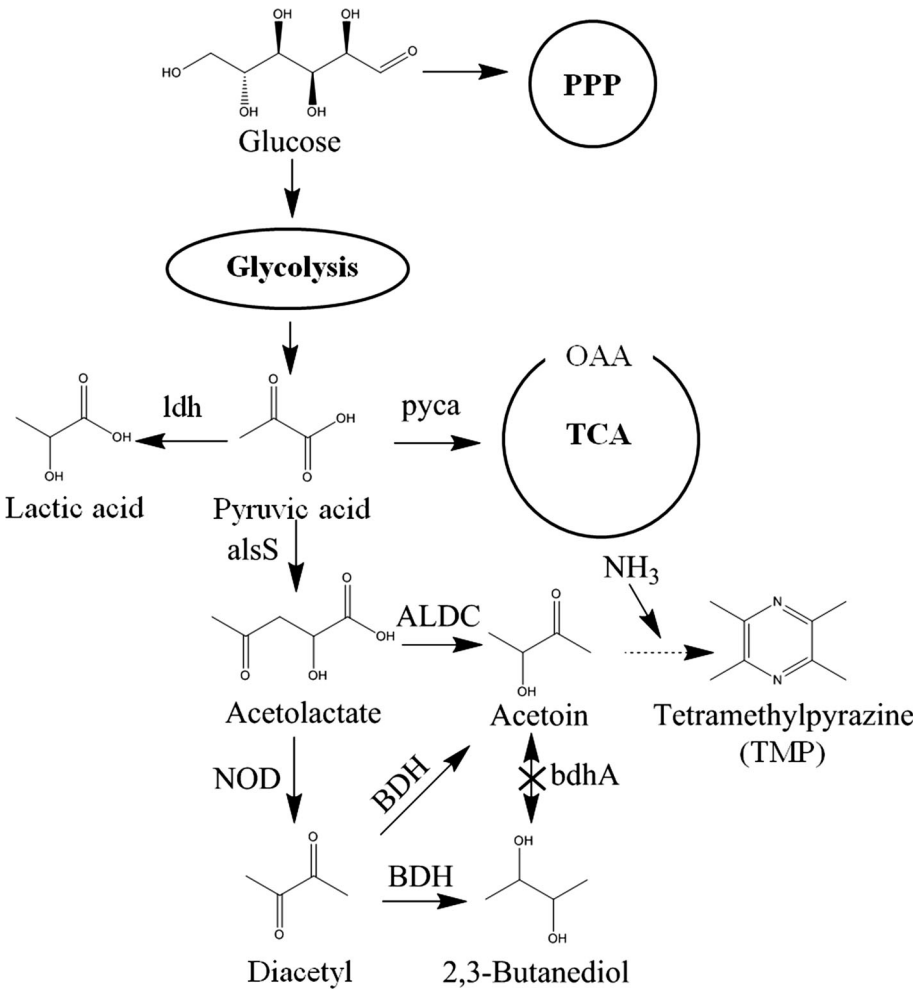

Fig. 2 Tetramethylpyrazine (TMP) biosynthetic pathway and other overflow metabolism pathways in *B. licheniformis* BL1. *AlsS*, *ALDC*, and *bdhA* encode acetolactate synthase, acetolactate decarboxylase, and 2,3-butanediol (2,3-BD) dehydrogenase, respectively. *PPP* pentose phosphate pathway, *TCA* tricarboxylic acid cycle, *NOD* Oxidative decarboxylation of non-enzymatic catalysis. Disrupted pathway steps are indicted by *arrow breaks*

(Fig. 2), accumulates and increases the yield of TMP. Compared with the acetoin yield of the parent strain (*Bacillus licheniformis* BL1), the acetoin yield of the *bdhA*-disrupted mutant strain (*B. licheniformis* BLA) increased by 28.98 % (w/w) from 15.22 to 19.63 g/l; the maximum TMP yield also increased by 23.99 % (w/w) from 37.89 to 46.98 g/l in the *bdhA*-disrupted mutant strain (*B. licheniformis* BLA). In contrast, 2,3-BD production decreased by 78.44 % (w/w) in the *bdhA*-disrupted mutant strain (*B. licheniformis* BLA) (Fig. 3).

Effect of 2,3-BD supplementation in the fermentation medium of BL1

The effect of 2,3-BD on TMP and acetoin production by BL1, was investigated by adding 1, 2, 3, and 4 g/l of 2,3-BD to the medium. The TMP production was influenced by the supplementation of 2,3-BD in the fermentation medium of BL1. The TMP and acetoin production improved when 1–3 g/l of 2,3-BD was added to the culture medium; this supplementation slightly influenced cell growth (Table 3),

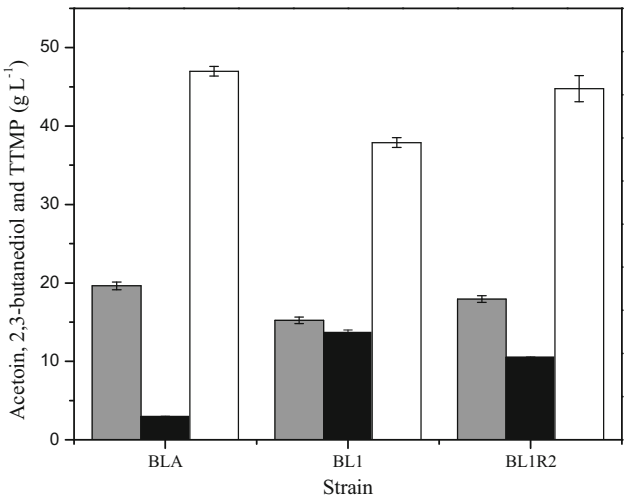

Fig. 3 The maximum acetoin (*gray bar*), 2,3-BD (*black bar*) and TMP (*blank bar*) concentrations of BL1, BLA and 2,3-BD-regulated BL1 (BL1R2) cultivated with 200 ml LB in 500 ml flask shaken at 200 rpm and 37 °C for 144, 72 and 168 h, respectively; data are average values and standard deviations of triplicate experiments

Table 3 Effect of 2,3-BD addition on TMP production by *B. licheniformis* BL1 (n = 3)

Cultivation conditions			2,3-BD addition ($g \cdot l^{-1}$)	Time[a] (h)	Yield of acetoin ($g\ l^{-1}$)	Time[b] (h)	Yield of TMP ($g\ l^{-1}$)	OD_{600}^{max}
Initial glucose concentration ($g\ L^{-1}$)	pH	T[c] (°C)						
70.0	7.5	37	0	120.1	15.22 ± 0.24	168.5	37.89 ± 0.40	44.78 ± 0.87
			1.0	120.4	16.65 ± 0.15	167.8	40.03 ± 0.58	42.88 ± 0.42
			2.0	120.5	17.94 ± 0.28	167.7	44.77 ± 0.66	23.52 ± 0.25
			3.0	120.3	16.04 ± 0.12	167.6	38.10 ± 0.37	23.74 ± 0.23
			4.0	119.4	14.53 ± 0.06	168.4	36.68 ± 0.12	22.89 ± 0.14

Data are average values and standard deviations of triplicate experiments

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

[b] Time in hours from inoculation to the maximal TMP concentration arrived

[c] *T*, temperature

residual glucose concentration, and 2,3-BD production by BL1 (Fig. 5). Tetramethylpyrazine (TMP) increased with an increase in the dosage of 2,3-BD, but no further increase was observed when the supplemented 2,3-BD in the matrix was ≥3.0 g/l (Table 3). Compared with those of BL1 (no dosage), the maximum TMP and acetoin yields increased by 18.16 % (w/w) and 17.87 % (w/w), respectively in BL1R2 (BL1 with 2 g 2,3-BD/l); this increase was observed when the strains were cultivated for 120 and 168 h, respectively (Fig. 3). In general, 2 g/l is an ideal initial 2,3-BD concentration that achieves maximum production of TMP and acetoin.

Discussion

Compared with those in BL1, the TMP and acetoin production in BLA increased by 23.99 % (w/w) and 28.98 % (w/w), respectively, when the strains were cultivated for 168 and 120 h, respectively (Fig. 3). This increase was mainly due to (1) the elimination of 2,3-BD production in the early stationary phase, and (2) the accumulation of the acetoin precursor in the early stationary phase. *B. licheniformis* BL1 accumulated more than 13 g/l of 2,3-BD when this strain was cultivated for 72 h. *bdhA*-disrupted mutant strain (*B. licheniformis* BLA) accumulated <3 g 2,3-BD/l when this strain was cultivated for 72 h in the medium with 0.96 g residual glucose/l though the amount of 2,3-BD decreased during the stationary phase (Fig. 4). This is consistent with a previous study that suggested a 2,3-BD degradation pathway where acetoin was an intermediate (Thanh et al. 2010).

As shown in Fig. 4, the amount of acetoin accumulated by BL1 began to decrease at 120 h, due to acetoin degradation. The utilization of acetoin by BL1 is mainly as a precursor for TMP or as a carbon source (Huang et al. 1999). The concentration of acetoin increases in BLA; as a result, there is an increase in TMP concentration. However,

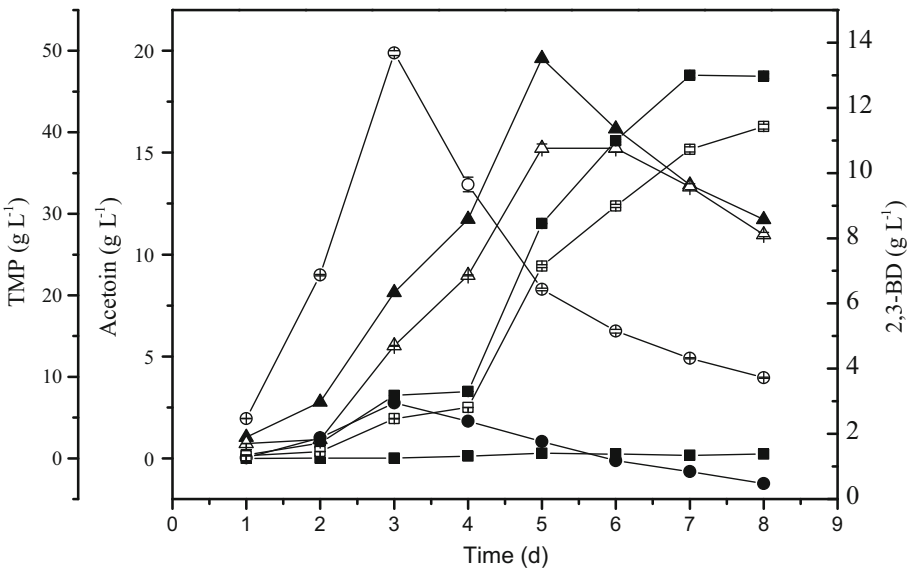

Fig. 4 Effects of *bdhA* disruption on acetoin, TMP and 2,3-BD production during the stationary phase of cultivation with 200 ml LB in 500 ml flask shaken at 200 rpm and 37 °C for 8 days; Product profiles of BL1 (*open*) and BLA (*filled symbols*) are shown. *Squares*, *triangles* and *circles* represent TMP, acetion and 2,3-BD concentrations of the same strain, respectively

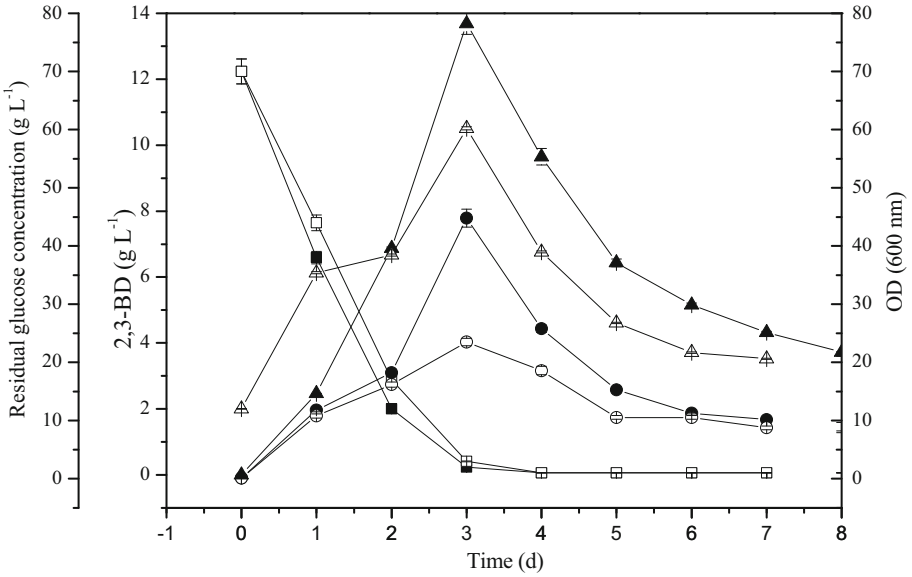

Fig. 5 Effect of 2,3-BD addition on cell growth (*filled circle*), 2,3-BD production (*filled triangle*) and residual glucose concentration (*filled square*)by *B. licheniformis* BL1. Product profiles of BL1 (*open*) and BL1R2 (*filled symbols*) are shown

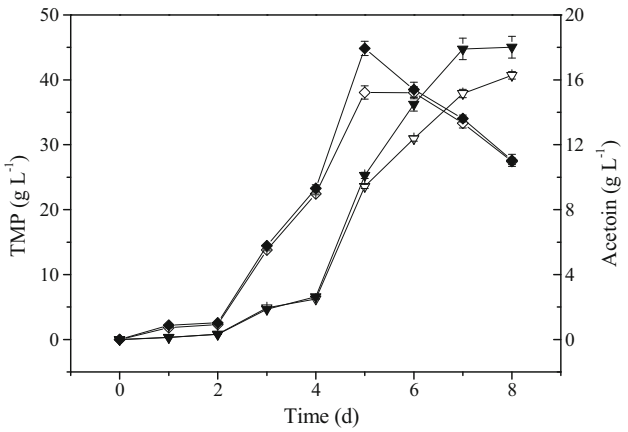

Fig. 6 Effect of 2,3-BD addition on acetoin production (*filled square*), TMP production (*filled triangle*) by *B. licheniformis* BL1. Product profiles of BL1 (*open*) and BL1R2 (*filled symbols*) are shown

acetoin is metabolized more slowly by BLA than by BL1 (Silbersack et al. 2006).

An interesting result was that the TMP and acetoin yields improved from 37.89 to 44.77 g/l and from 15.22 to 17.94 g/l, respectively, as the 2,3-BD dosage increased from 0 to 2 g/l in the BL1 medium. Thus, 2,3-BD can promote the TMP and acetoin yields in a dose-dependent manner. The maximum TMP and acetoin yields was obtained with a concentration of 2 g 2,3-BD/l in the culture medium. The results suggest this methodology is a cost-effective option for TMP and acetoin production. Nevertheless, the mechanism by which acetoin is improved by 2,3-BD supplementation is poorly understood. Acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH) likely catalyzes the conversion between acetoin (AC) and 2,3-butanediol (BD) (Juni and Heym 1956); The initial 2,3-BD has a feedback effect on the catalytic conversion of BD to AC, which raises the concentration of acetoin and lowers the concentration of BD (Fig. 5). After 2,3-BD is added, there is an accumulation of the precursor acetoin in BL1 and hence an increased production of TMP (Fig. 6). 2,3-butanediol (2,3-BD) is slightly conducive to cell growth (Fig. 5). Enzyme activities reach a peak in the presence of high carbohydrate concentrations and rapidly decrease after the carbon sources are depleted (Skory 2000). Thus, the initial suppression of AR/BDH by 2,3-BD causes a very low activity and impedes the synthesis of 2,3-BD when the sugars are almost completely exhausted at the end of the fermentation. Further, there is a report that 2, 3-BD can continue to serve as a carbon source to generate acetoin when the concentration of carbon source is low in the medium (Zhang et al. 2011). However, when a specific inhibitory concentration of 2,3-BD is added, cell growth is adversely affected and product synthesis is inhibited (Table 3). The 2,3-BD inhibitory mechanism is not yet clear. This is probably due to certain enzymes that are activated or inhibited by 2,3-BD in the synthesis or metabolism process, or the transfer process is induced by different kinds of enzymes. Therefore, the inhibitory mechanism of 2,3-BD needs to be further investigated, and related analyses are required.

Acknowledgments This work was supported by National High Technology Research and Development Program of China (863 Program) (Grant No. 2012AA022108) and The Science and Technology Development Plan Project of Shan dong Province (Grant No. 2014GSF121008).

References

Ai-ping Z, Di-xun W, Feng W (1986) Effect of tetramethylpyrazine on acute and chronic hypoxic pulmonary hypertension of the rat. J Tongji Med Univ 6:133–137

Amrani-Hemaimi M, Cerny C, Fay LB (1995) Mechanisms of formation of alkylpyrazines in the maillard reaction. J Agric Food Chem 43:2818–2822

Fan W, Xu Y, Zhang Y (2007) Characterization of pyrazines in some chinese liquors and their approximate concentrations. J Agric Food Chem 55:9956–9962

Gao S, Guo W, Shi L, Yu Y, Zhang C, Yang H (2014) Characterization of acetoin production in a budC gene disrupted mutant of *Serratia marcescens* G12. J Ind Microbiol Biotechnol 41:1267–1274

Hao F, Wu Q, Xu Y (2013) Precursor supply strategy for tetramethylpyrazine production by *Bacillus subtilis* on solid-state fermentation of wheat bran. Appl Biochem Biotechnol 169:1346–1352

Huang M, Oppermann-Sanio FB, Steinbuchel A (1999) Biochemical and molecular characterization of the *Bacillus subtilis* acetoin catabolic pathway. J Bacteriol 181:3837–3841

Jianfeng W, Yan X (2014) Formation mechanism of tetramethylpyrazine produced with *B. subtilis* S12 under the fermentation condition simulated bacterial qu preparation used for chinese liquor brewing. J Food Sci Biotechnol 33

Juni E, Heym GA (1956) A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethyl carbinol and diacetyl II.: the synthesis of diacetylmethylcarbinol from diacetyl, a new diphosphothiamin catalyzed reaction. J Bacteriol 72:746–753

Kosuge T, Adachi T, Kamiya H (1962) Isolation of tetramethylpyrazine from culture of *Bacillus* natto, and biosynthetic pathways of tetramethylpyrazine. Nature 195:1103

Lancker FV, Adams A, Kimpe ND (2010) Formation of pyrazines in maillard model systems of lysine-containing dipeptides. J Agric Food Chem 58:2470–2478

Lancker FV, Adams A, Kimpe ND (2011) Chemical modifications of peptides and their impact on food properties. Chem Rev 111:7876–7903

Lancker FV, Adams A, Kimpe ND (2012) Impact of the N-terminal amino acid on the formation of pyrazines from peptides in maillard model systems. J Agric Food Chem 60:4697–4708

Muller R, Rappert S (2010) Pyrazines: occurrence, formation and biodegradation. Appl Microbiol Biotechnol 85:1315–1320

Sabik H, Fortin J, Martin N (2012) Identification of pyrazine derivatives in a typical maple syrup using headspace solid-phase microextraction with gas chromatography–mass spectrometry. Food Chem 133:1006–1010

Shi L, Gao S, Yu Y, Yang H (2014) Microbial production of 2,3-butanediol by a newly-isolated strain of *Serratia marcescens*. Biotechnol Lett 36:969–973

Silbersack J, Jürgen B, Hecker M, Schneidinger B, Schmuck R, Schweder T (2006) An acetoin-regulated expression system of *Bacillus subtilis*. Appl Microbiol Biotechnol 73:895–903

Skory CD (2000) Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae*. Appl Environ Microbiol 66:2343–2348

Thanh T, Jurgen B, Bauch M, Liebeke M, Lalk M, Ehrenreich A, Evers S, Maurer K-H, Antelmann H, Ernst F, Homuth G, Hecker M, Schweder T (2010) Regulation of acetoin and 2,3-butanediol utilization in *Bacillus licheniformis*. Appl Microbiol Biotechnol 87:2227–2235

Xiao ZJ, Xie NZ, Liu PH, Hua DL, Xu P (2006) Tetramethylpyrazine production from glucose by a newly isolated *Bacillus mutant*. Appl Microbiol Biotechnol 73:512–518

Yan X, Qun W, Wenlai F, Bingfeng Z (2011) The discovery and verification of the production pathway of Tetramethylpyrazine(TMP) in chiese liquor. Liquor-Making Sci Technol 7:37–40

Zhang X, Yang T, Lin Q, Xu M, Xia H, Xu Z, Li H, Rao Z (2011) Isolation and identification of an acetoin high production bacterium that can reverse transform 2,3-butanediol to acetoin at the decline phase of fermentation. World J Microbiol Biotechnol 27:2785–2790

Zhu B-F, Xu Y, Fan W-L (2010) High-yield fermentative preparation of tetramethylpyrazine by *Bacillus* sp. using an endogenous precursor approach. J Ind Microbiol Biotechnol 37:179–186