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ORIGINAL PAPER

High-yield fermentative preparation of tetramethylpyrazine by *Bacillus* sp. using an endogenous precursor approach

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Abstract A spore-forming *Bacillus* sp. was isolated from a high-temperature Daqu, a starter culture of Chinese *Maotai-flavor* liquor, using an endogenous precursor screening strategy. The *Bacillus* sp. was capable of producing a high level of 2,3,5,6-tetramethylpyrazine (TTMP) via a precursor of 3-hydroxy-2-butanone (HB). The strain was characterized as *Bacillus subtilis* based on morphological, physiological, and biochemical properties as well as on partial 16S rRNA gene sequences. Different carbon and nitrogen sources as well as fermentation conditions were investigated. Optimization tests showed that oxygen supply and fermentation temperature were the most important parameters determining the production process. The production of >4.08 g/l TTMP was achieved together with a high level of endogenous precursor HB accumulation (>20 g/l) in both flask and fermentor cultures when the optimized medium and cultivation conditions were applied. Our data demonstrates the effectiveness of the endogenous precursor strategy for screening microorganisms that produce flavor compounds with structure-related precursors. The high yield of TTMP and the inexpensiveness of the agro-industrial product used as the substrate (soybean meal) indicate the potential of this process for industrial application.

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Introduction

Alkylpyrazines are a group of heterocyclic nitrogen-containing compounds that are ubiquitous in both raw and processed food and alcoholic beverages [6]. They are generally considered to be important aroma compounds that give tonalities of nutty, roasty, and toasty [19]. 2,3,5,6-Tetramethylpyrazine (TTMP) is a commonly occurring alkylpyrazine, and this compound has been found to be responsible for the characteristic odor of oriental food, such as *natto*, a Japanese fermented soybean product [14]. Recently, TTMP in combination with other alkylpyrazines has also been identified in Chinese liquors and, based on flavor dilution values, it has been suggested that these alkylpyrazines may be the main contributors to the *Maotai-flavor* of these liquors [6].

In addition to having flavoring additive properties, TTMP, as the main bioactive ingredient of alkaloids isolated from the rhizome of *Ligusticum wallichii*, has also been proven to have pharmacological activity and to be both efficacious for cardiac and cerebrovascular disease [8] and have protective effects on cisplatin-induced oxidative stress, apoptosis, and nephrotoxicity [18].

To date, numerous methods of pyrazine synthesis via the Maillard reaction and Strecker degradation have been established [2, 11], but the involvement of chemical mediators leads to the less appreciated label of ‘artificial’ compared with ‘nature’ or ‘bio’ products [26]. The direct extraction of TTMP from *L. wallichii* plant is often associated with a low concentration of the TTMP (approximately

0.075%, wt/wt) as endproduct, limited availability of substrate sources, and high extraction costs, all of which hamper the processing of TTMP on an industrial scale [16, 17]. The disadvantages of both of these methods combined with the increasing interest in natural products suggest that the biotechnological production of pyrazines is a promising and effective approach.

TTMP was initially isolated from a culture of *Bacillus natto* [14], and several subsequently approaches to pyrazine production have been explored in liquid-state, solid-state, and submerged fermentations using *Bacillus* sp. [3, 15, 28], a *Corynebacterium glutamicum* mutant [5], and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* FC1 [13]. Although many microorganisms have the metabolic potential to biosynthesize de novo TTMP, and TTMP can be detected in microbial cultures, the concentrations produced are usually too low to be employed for commercial applications. The addition of an exogenous precursor to the culture system has been a commonly employed approach to improve the yield of TTMP [3, 15], such as the exogenous addition of quantities of precursor 3-hydroxy-2-butanone (HB) to culture medium during microbial cultivation. Such exogenous supplements have proven to be an effective way to enhance TTMP production.

However, the exogenous precursor approach for enhanced TTMP production generally requires sufficient amounts of exogenously added precursor HB during fermentation, and this usually results in metabolic imbalance with product feedback inhibition and cell toxicity [26]. Even more disappointedly, even though a higher pyrazine production was achieved using HB applied exogenously, the precursor conversion rate was low [15]. A further bottleneck in using this approach is the difficulty in obtaining sufficient amounts of the precursor HB in an economically viable manner from natural sources. Consequently, the screening of microbes with enhanced metabolic flux to precursor HB excretion endogenously from glucose or other carbon sources is essential for the high production of TTMP. The method for enhanced flavor production via endogenous structure-related precursor accumulation is called the endogenous precursor approach.

To the best of our knowledge, most microbial producers of alkylpyrazines in Oriental fermented food are spore-forming *Bacillus* sp. *Bacillus* sp. are also abundant in Daqu, the starter culture of Chinese *Maotai-flavor* liquors. The presence of high concentrations of pyrazines in *Maotai-flavor* liquors indicates that the liquor-making fermentation environment is beneficial to pyrazine formation. We therefore chose high-temperature Daqu as the mixed fermentation product to be used as the bacterial material for screening TTMP-producing microbes.

We report here an endogenous precursor strategy for screening TTMP-producing microorganisms from a high-temperature Chinese *Maotai-flavor* Daqu. The influence of different fermentation parameters on microbial TTMP production was investigated, and the potential of the combination of optimized medium and cultivation conditions for industrial applications were verified in both flask and fermentor experiments.

Materials and methods

Microorganisms, medium, and culture conditions

Microorganisms were isolated from the high-temperature *Maotai-flavor* Daqu donated by companies in Kweichow Moutai area (China). Solid Daqu was ground into powder and stored at 4°C until use.

Samples of this high-temperature Daqu powder (5 g) were shaken for 1 h with 25 ml sterile deionized water in an 100-ml flask. The cell suspension was then heat treated in a water bath at 80°C for 20 min. For the enrichment of the microorganisms, 1% inocula of Daqu cell suspension was inoculated into 20 ml basal medium [(g/l) glucose, 10; beef extract, 5; g NaCl, 5; initial pH 7.2] in an 100-ml flask at 30°C for 24 h with agitation; the culture was then successively sub-cultured (1:10 dilution in every transfer) six times, spread on nutrient agar [(g/l) glucose, 10; fish peptone, 10; beef extract, 5; NaCl, 5; agar, 15; initial pH 7.2], and incubated at 30°C for 48 h. Pure cultures with different morphologies were obtained and inoculated on nutrient agar for further study.

Flask experiments were carried out in 250-ml Erlenmeyer flasks containing 50 ml modified PYG medium [(g/l) glucose, 100; fish peptone, 30; yeast extract, 10; diammonium phosphate, 30; pH 7.2 before autoclaving] that were incubated at 30°C with shaking (150 r/min) for 48 h. Glucose was autoclaved separately for 15 min at 121°C.

Screening of HB- and TTMP-producing strains

Pure cultures were incubated in basal medium at 30°C for 48 h with shaking (150 r/min), and the fermentation broth was then assayed for HB-forming ability by the Voges–Proskauer (V–P) test [20] with some modifications. In brief, 100 µl creatine (3% in water, w/v) and 1 ml NaOH (40%, w/v) were added sequentially to 1 ml of the appropriately diluted fermentation broth; the samples were vortexed after each addition and then incubated at 30°C for 15 min, following which they were vortexed again before the maximum absorbance was measured at a wavelength of 560 nm (A_{560}) in a spectrophotometer.

Pure cultures which gave higher A_{560} values were selected and incubated in modified PYG medium. The concentrations of TTMP and precursor HB in the fermentation

broth were assayed by gas chromatography (see Analytical Methods).

Effect of carbon and nitrogen sources on TTMP production

To test the effect of carbon sources on TTMP production, we cultured the isolated microorganisms on medium containing 30 g/l diammonium phosphate, 30 g/l of peptone, and 10 g/l yeast extract supplemented with 100 g/l different carbon sources (glucose, sucrose, fructose, maltose, dextrin, or soluble starch). To determine the influence of nitrogen sources on TTMP production, the cultures were carried out on the medium containing 30 g/l diammonium phosphate, 100 g/l of glucose, and 40 g/l different nitrogen sources (fish peptone, yeast extract, tryptone, soybean meal powder, corn steep liquor, beef extract, or malt powder). In all of these experiments, the carbon sources were autoclaved separately for 15 min at 121°C. Erlenmeyer flasks (volume 250 ml), each containing 50 ml of the respective medium, were incubated at 30°C for 96 h with shaking (150 r/min). All experiments were performed in triplicate and the mean determined.

Effect of culture conditions on TTMP production

We investigated the effects of initial medium pH, oxygen supply, and cultivation temperature on the TTMP produced by *B. subtilis*. To determine the initial optimum pH, we adjusted the modified PYG medium with 10 mol/l NaOH or 10 mol/l HCl at pH ranging from 5.5 to 8.5, respectively. The effects of oxygen supply on TTMP production were tested in 50 ml medium in 250-ml flasks with shaking at 120, 150, or 200 r/min, respectively. Different incubation temperatures (30, 32, 35, 37, or 40°C) were also tested for their effect on TTMP production in the modified PYG medium with shaking at 200 r/min. All experiments were performed in triplicate and the mean determined.

TTMP production in pH-uncontrolled batch fermentation

The potential of the optimized medium combination was verified in 7-l fermentor (BioFlo 110; New Brunswick Scientific, Edison, NJ) experiments. Batch cultures were conducted in 4 l of culture medium [(g/l) sucrose, 100; soybean meal, 40; diammonium phosphate, 30] supplemented with 5 g/l yeast extract; the initial pH was adjusted to 7.5. The isolated strain *B. subtilis* XZ1124 was pre-cultured in modified PYG medium for 20 h with shaking (200 r/min) at 37°C before being inoculated (2.5%, v/v) into the fermentor. The fermentor was run at 37°C with stirring (500 r/min); air was supplied at a flow rate of 1.0 vvm, and the pH was uncontrolled during fermentation.

Analytical methods

Total viable cells and heat-resistant spores (80°C, 10 min) were measured by counting the colony-forming units (CFU) as previously described [4]. Fermentation broth was harvested by centrifugation (10,000 *g*, 10 min, 4°C), and the supernatant was diluted to appropriate folds with phosphate buffer (0.5 mol/l, pH 7.0). The dilutions were then assayed for substrates and products. Total sugar content was estimated using the DNSA method [21]. TTMP and HB in the dilutions were extracted by dichloromethane with the addition of 2-heptanone as the internal standard. The organic phase was directly analyzed using an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector (FID) and a 30-m DB-Wax capillary column (0.32 mm inside diameter, 0.25 μm film thickness). The operating conditions were as follows: nitrogen was used as the carrier gas; the injector temperature and the detector temperature were both at 250°C; the column oven was kept constant at 80°C for 2 min, then programmed to 210°C with a temperature increase of 10°C/min.

Results

Screening of TTMP-producing strains

About 300 pure cultures with different morphologies were obtained. Heating treatment of the Daqu bacterial suspensions at 80°C for 20 min proved to be an effective way to isolate mesophilic spore-formers, including *Bacillus* and *Clostridium* species.

The ability of the bacteria to produce HB from glucose was tested using the V–P colorimetric assay. Among the approximately 300 pure cultures isolated from the high-temperature Daqu, more than 100 gave higher A_{560} values. The ability of the bacteria to produce TTMP was then tested by inoculating the pure cultures into modified PYG medium. TTMP was detected in most HB-forming cultures, and the pure culture with the highest concentration of TTMP was selected for further study.

The fermentation products were also analyzed by mass spectrometry, and their spectra matched well with the spectra of TTMP and HB, respectively, from the mass spectrometry data library (data not shown).

Characterization of the isolated strain

The isolated strain was a rod-shaped, spore-forming bacterium that shared the typical characteristics of *Bacillus* species, i.e., it was Gram-positive, aerobic, catalase-positive, V–P test-positive, nitrate reduction-positive, and

lactate-positive. The nucleotide sequence of the 16S rRNA gene was obtained (GenBank accession number EU883786) and was compared to those in the National Center for Biotechnology Information nucleotide sequence database using the BLAST algorithm. The isolated strain showed a significant identity (>99%) to a number of strains of the Gram-positive bacteria *Bacillus subtilis*. This newly isolated *Bacillus* sp. was designated as *B. subtilis* XZ1124 and deposited in the China Center for Type Culture Collection (CCTCC M 208157).

Effect of carbon source on the production of TTMP

Among all of the carbon sources tested in this study, sucrose resulted in the best production of TTMP, 1.25 g/l, while maltose performed poorly in terms of both TTMP and HB accumulation (Fig. 1a). Glucose and fructose produced acceptable levels of TTMP with corresponding high concentrations of precursor HB. The difference between the sugars in terms of TTMP and HB accumulation indicates the selectivity of *B. subtilis* XZ1124 in utilizing carbon sources to biosynthesize desired flavor compounds.

Effect of nitrogen source on TTMP production

Fish peptone, tryptone, soybean meal, yeast extract, corn steep liquor, maltose powder, and beef extract were each screened as sole organic nitrogen source (40 g/l) for their effect on TTMP production. The results show that lower levels of TTMP were obtained with fish peptone, beef extract, and maltose powder (0.24, 0.26, and 0.32 g/l, respectively) (Fig. 1b). Higher yields of TTMP were found in the cultures supplemented with soybean meal and yeast extract—0.69 and 0.62 g/l TTMP, respectively; considerably higher yields of precursor HB were also obtained with these two nitrogen sources (15.2 and 10.5 g/l, respectively) than with the other organic nitrogen sources tested.

Since the scope of this study was to formulate low-cost media, soybean meal as an agro-industrial product possessing the advantages of high digestibility, high energy content, and consistency was preferred over fish peptone as a favorable nitrogen source. Yeast extract, which is rich in B vitamins, was also found to favor TTMP production when used as sole organic nitrogen source. We therefore investigated the interactive effect of the yeast extract and soybean meal on TTMP production in a full factorial design experiment with single-replicate treatment combinations of these two factors. The design matrix of the experiments and corresponding TTMP and precursor HB production are given in Table 1. The results show that 40 g/l soybean meal supplemented with 5 g/l yeast extract gave the best TTMP and HB production; this combination proved to be an effective complex nitrogen source that supported TTMP production by *B. subtilis* XZ1124.

Effect of culture conditions on TTMP production

The effect of varying initial pH values on TTMP production is shown in Fig. 1c. The concentration of TTMP was low in the culture with an initial pH of 5.5 and increased with increases in the initial pH towards neutrality. Although the highest amount of HB was observed with an initial pH of

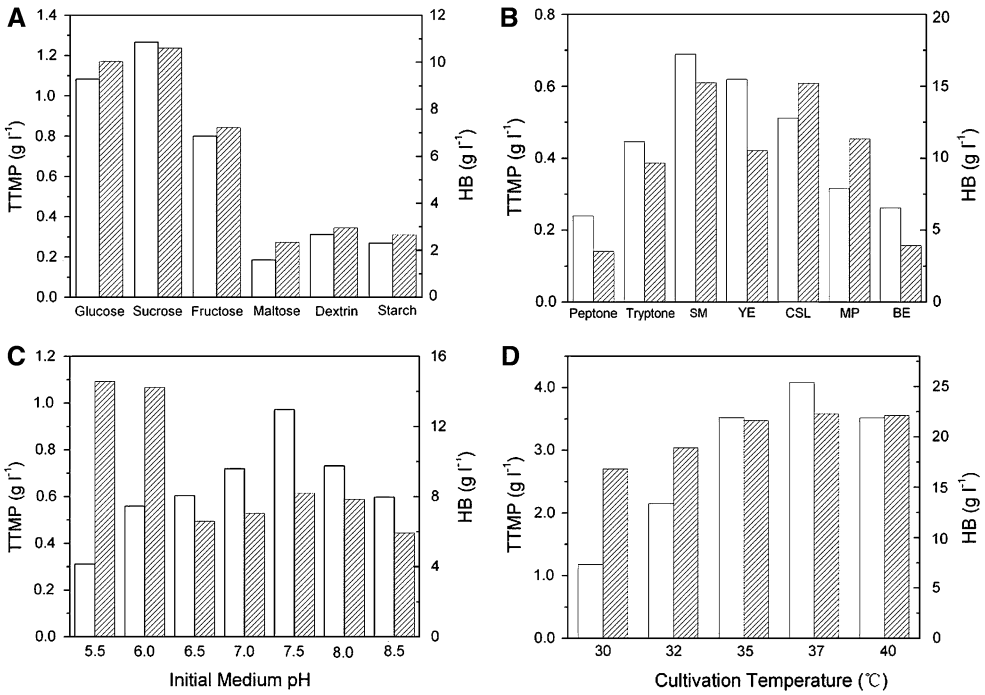

Fig. 1 Effects of carbon sources (**a**), organic nitrogen sources (**b**), initial medium pH (**c**), and cultivation temperature (**d**) on 2,3,5,6-tetramethylpyrazine (*TTMP*) and precursor 3-hydroxy-2-butanone (*HB*) production by *Bacillus subtilis* XZ1124. *Open columns* TTMP, *shaded columns* HB, *SM* soybean meal, *YE* yeast extract, *CSL* corn steep liquor, *MP* maltose powder, *BE* beef extract. Fermentation conditions are described in the text. Data were means of triplicates. Standard errors are less than 5.0% of the means

Table 1 Effects of complex nitrogen sources (soybean meal × yeast extract) on TTMP production

Soybean meal (g/l)	Yeast extract (g/l)	Final pH	TTMP (g/l)	HB (g/l)
20	5	6.31	0.48	11.8
20	10	6.31	0.51	13.2
20	15	6.44	0.54	15.0
30	5	6.33	0.54	11.8
30	10	6.39	0.68	20.3
30	15	6.54	0.63	16.3
40	5	6.22	1.04	23.3
40	10	6.36	0.80	20.0
40	15	6.48	1.06	21.9

TTMP, 2,3,5,6-Tetramethylpyrazine; HB, 3-hydroxy-2-butanone

Values in the first two columns are the concentrations of the respective nitrogen source tested in the culture medium (100 g/l sucrose, 30 g/l diammonium phosphate, and complex nitrogen source). Values in the fourth and fifth columns are the yields in TTMP and HB givens as the means of duplicate experiments. Standard errors were less than 5.0% of the means

Culture conditions were 37°C with shaking at 200 r/min

5.5, maximum TTMP production was found at an initial pH of 7.5. Oxygen supply had a significant effect on the performance of the fermentation process. The culture with a higher oxygen transfer rate (rotation speed 200 r/min) produced higher levels of TTMP and precursor HB (1.18 and 18.8 g/l, respectively); in comparison, the production of TTMP and precursor HB was only 0.17 and 6.6 g/l, respectively, at a lower oxygen transfer rate (rotation speed 120 r/min). The efficiency of the fermentation process was strictly temperature dependent owing to the strong dependence of enzymatic activity and cellular maintenance on temperature. Increases in the culture temperature from 30 to 37°C resulted in significant enhancements of TTMP and HB production (2.5-fold and 33% improvement, respectively) (Fig. 1d). When the fermentation temperature increased above 37°C, cell degradation probably became dominant over the growth process. This would result in a breakdown in cellular regulatory mechanisms related to metabolism, with a resultant decrease in TTMP production; HB yield, however, would remain essentially constant (Fig. 1d).

Time course of TTMP production in flask fermentation

To investigate the dynamic model for TTMP production, flask experiments were performed under the optimized culture conditions using the optimized medium. As shown in Fig. 2, the number of viable cells increased rapidly 8 h post-culture initiation and reached the stationary phase at 48 h. The precursor HB concentration reached its maximum value (22.3 g/l) at 48 h and ceased to increase as cell growth ended, exhibiting a linear correlation with the increasing number of viable cells. This observation suggested that HB secreted by *B. subtilis* XZ1124 is a typical growth-associated product as a primary metabolite from sugars.

The concentration of total sugar kept declining until the number of viable cells began to decrease. Nearly no TTMP was detected in the first 20 h post-culture initiation, while it began to accumulate rapidly at the middle of the logarithmic phase, increasing linearly to 4.08 g/l at 112 h. A slight decrease in TTMP level was observed at 120 h of cultivation, probably due to the combined effect of decreased precursor HB and viable cell number as well as product evaporation. Spores formed slowly in the logarithmic phase and stationary phase, and higher spore numbers were only found late in the culture period as the total number of viable cells began to decrease. The pH decreased rapidly during the first 16 h post-culture initiation, remained constant in the logarithmic phase, and decreased slowly late in the culture period.

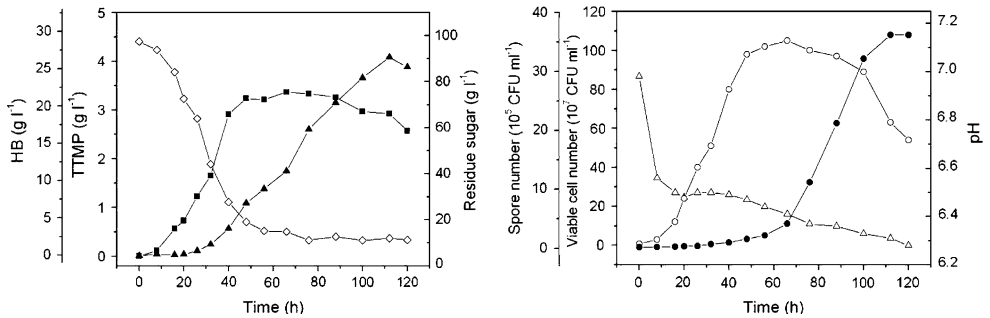

Fig. 2 Time course of TTMP fermentation in flask experiments under the optimized medium and cultivation conditions by *B. subtilis* XZ1124. The culture medium consisted of 100 g/l sucrose, 40 g/l soybean meal, and 30 g/l diammonium phosphate supplemented with 5 g/l yeast extract; the initial pH was adjusted to 7.5. The medium was then cultivated in 250-ml flasks at 37°C with shaking at 200 r/min. *Filled triangle* TTMP, *filled square* HB, *open circle* total viable cell number, *open triangle* pH, *filled circle* spore number, *open diamond* residue sugar. Data are the means of triplicate experiments. Standard errors are less than 5.0% of the means

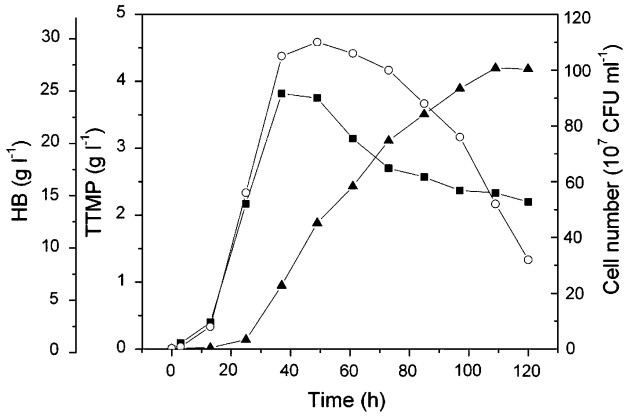

Fig. 3 Time course of TTMP fermentation in 7-l fermentor by *B. subtilis* XZ1124. *Filled triangle* TTMP, *filled square* HB, *open circle* viable cell number. Fermentation medium and cultivation conditions were shown in the text. Data were means of triplicate experiments. Standard errors are less than 5.0% of the means

Verification of the optimized medium combination in the 7-l fermentor

To verify the potential of the optimized medium combination, we carried out a re-check of our experimental findings in a 7-l fermentor (4-l working volume). A mean value of 4.20 g/l TTMP was obtained, which adequately confirmed the potential of this newly isolated strain for industrial application. TTMP, precursor HB, and cell growth were monitored (Fig. 3): increases in the production of both TTMP and precursor HB were observed, probably due to the elevation of oxygen supply rate in the fermentor environment.

Discussion

HB is an important physiological metabolic product secreted by various microorganisms, and its function as the precursor of TTMP has been verified in several studies [3, 13, 15, 24]. Although the production of TTMP in systems applying the exogenous precursor approach has been effective for obtaining the desired flavor compounds, the biocatalytic conversion of the HB molecule derived from primary metabolic pathways functioning in situ (endogenous precursor approach) has been shown to be a superior strategy for stimulating TTMP accumulation. In our study, the production of 4.08 g/l TTMP (near the solubility limit of 4.77 g/l at 37°C [28]) was obtained in a system in which a precondition was 19.4 g/l precursor HB accumulation under optimized culture conditions. The screening strategy used here is also applicable for screening microbes producing other flavor compounds with structure-related precursors.

We observed that a higher oxygen supply favored the accumulation of precursor HB and TTMP. Moes et al. [22] showed that the dissolved oxygen level had a profound effect on the product distribution of a *B. subtilis* culture. Therefore, in our study, changes in the oxygen level could have caused HB to be converted to 2,3-butanediol in a reversible manner. Similarly, Nakashimada et al. [23] increased HB formation with a high air supply when investigating the effect of oxygen supply on diol fermentation by *Paenibacillus polymyxa* ATCC 12321 (formerly classified as a member of the *Bacillus* genus). Tetramethyldihydropyrazine has been characterized as an intermediate in the transformation of the corresponding TTMP in a redox reaction [10]; as such, this reaction may be accelerated by an increased dissolved oxygen level.

In our study, sporulation mainly occurred in the late culture period, after the total number of viable cells began to decrease at 66 h post-culture initiation. In this same period (>66 h), TTMP concentration increased linearly, and precursor HB concentration began to decrease from its maximum value (22 g/l); cell lysis was also observed in this period. Based on this observation that cell sporulation was accompanied by increases in TTMP concentration during the late culture period, we speculate that TTMP may be a secondary metabolite related to the sporulation cycle. The increases in spore number during this period can also be explained as the survival of strategy of *B. subtilis* XZ1124 under adverse culture conditions: the cytotoxicity of high concentrations of precursor HB and increasing accumulation of TTMP in the culture environment is usually a major bottleneck during bioprocess development [26]. The hydrophobic character of TTMP resulted in these molecules accumulating preferentially in the cellular lipid bilayer membrane, which induced enhanced membrane fluidity, and eventually led to collapsing transmembrane gradients and, consequently, to the loss of cell viability [27].

Despite the metabolic flux to TTMP formation, there was an increased reduction in precursor HB levels after 88 h post-culture initiation. One reason for this may be the physiological function of HB, which is an energy-storing material derived from pyruvate [7, 12]. In *B. subtilis*, HB breakdown is mainly catalyzed by the acetoin dehydrogenase enzyme system (AoDH ES) [9], and the aco operon encoding AoDH ES is subject to direct and indirect CcpA-dependent glucose transcriptional repression [29]. In the presence of abundant glucose during the early culture period, the synthesis pathway of HB as primary metabolite was activated, and the corresponding catabolic pathways were transcriptionally repressed. However, when the glucose became depleted in the late culture period, the repression disappeared, and HB was consumed as the successive energy and carbon source to keep the cell active.

The mechanism of microbial TTMP formation has not yet been fully explored. Although the hypothesis that microbial TTMP is synthesized from acetoin and ammonia was proposed by Adachi [1] and has been supported by several studies [3, 5, 13, 15, 28], the related enzymes which catalyze the reactions in which the precursor HB is transformed into TTMP have not yet characterized. Pyrazine formation by nonenzyme-catalyzed reactions of acyloins with ammonia under mild conditions has also been demonstrated [25]. Future research efforts in this field should be focused on the mechanism of TTMP biosynthesis from HB by *B. subtilis* in the fermentation environment.

In summary, an endogenous precursor strategy for screening microorganisms which produce flavor compound producers with structure-related precursors is reported here. This screening strategy was used on Chinese high-temperature *Maotai-flavor* Daqu, and a *B. subtilis* XZ1124 was isolated and identified. The newly isolated strain was found to be capable of producing a high level of TTMP (>4.08 g/l) with a high accumulation of the endogenous precursor HB in both flask and fermentor cultures. Preliminary optimization experiments revealed that neutral pH, good oxygen supply, and a suitable culture temperature were necessary for better precursor HB accumulation leading to a high production of TTMP. Soybean meal could be utilized as a preferable nutrient by food-grade *B. subtilis* XZ1124. This strain possesses promising characteristics for utilization in an economically feasible and environmentally acceptable fermentation process with the potential for industrial applications.

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