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ORIGINAL PAPER

Production of tetramethylpyrazine by batch culture of *Bacillus subtilis* with optimal pH control strategy

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Abstract The effects of initial culture pH ranging from 5.0 to 7.5 on biomass content, precursor 3-hydroxy-2-butanone (HB) accumulation, and 2,3,5,6-tetramethylpyrazine (TTMP) formation by *Bacillus subtilis* CCTCC M 208157 were investigated in shake flask fermentation. Weak acidic conditions were found to favor cell growth and precursor HB accumulation, while TTMP could be synthesized more efficiently in conditions with initial pH towards neutrality. Batch bioprocess of TTMP fermentation by *Bacillus subtilis* CCTCC M 208157 at various controlled pH values ranging from 5.5 to 7.0 was then examined in 7.5-l fermentor. The results suggested that optimum pH for cell growth and precursor HB accumulation was 5.5 with maximum cell growth rate (Q_x) and precursor HB accumulation rate (Q_{HB}) of 0.833 g l^{-1} h^{-1} and 1.118 g l^{-1} h^{-1}, respectively, while optimum pH for TTMP formation was 7.0 with maximum TTMP formation rate (Q_{TTMP}) of 0.095 g l^{-1} h^{-1}. A pH-shifted strategy was accordingly developed to improve TTMP production in bioreactor fermentation by shifting the culture pH from 5.5 to 7.0 after 48 h of cultivation. By applying the strategy, final TTMP concentration of 7.43 g l^{-1} was obtained, being 22.2% greater than that of constant-pH fermentation.

Keywords Tetramethylpyrazine · Precursor · *Bacillus subtilis* · Batch fermentation · pH strategy

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List of symbols

P_{HBmax}	Maximal precursor HB concentration (g l^{-1})
P_{TTMP}	Final product (TTMP) concentration (g l^{-1})
X_{max}	Maximal dry cell weight (biomass) (g l^{-1})
Q_x	Cell production rate (g l^{-1} h^{-1})
Q_{HB}	Precursor HB formation rate (g l^{-1} h^{-1})
Q_{TTMP}	Product (TTMP) formation rate (g l^{-1} h^{-1})
q_{HB}	Specific precursor HB accumulation rate (h^{-1})
q_{TTMP}	Specific product (TTMP) formation rate (h^{-1})
$Y_{HB/x}$	Precursor HB yield from biomass (g g^{-1})
$Y_{TTMP/HB}$	Product (TTMP) yield from precursor HB (g g^{-1})
$Y_{x/s}$	Cell yield from substrate (g g^{-1})

Introduction

2,3,5,6-Tetramethylpyrazine (TTMP, Ligustrazine), one of the commonly occurring alkylpyrazines, is widely used in the food industry as a flavor ingredient [16] for nutty, roasted, and toasted tonalities [13]. As the main bioactive ingredient of alkaloids isolated from the rhizome of *Ligusticum wallichii*, TTMP has also been proved to have pharmacological effects in clinical applications [6].

Although numerous methods of pyrazine synthesis via Maillard reaction and Strecker degradation have been developed in recent decades [2, 8], there has been increasing interest in microbial TTMP production from natural raw materials for its "bio" or "natural" properties [15]. Several microorganisms including *Bacillus* sp. [3, 10, 18], a *Corynebacterium glutamicum* mutant [4], and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* FC1 [9] were shown to have metabolic potential to biosynthesize

TTMP. However, attempts to use these microorganisms on commercial scale were usually hampered by low concentration of TTMP in the fermentation broth, resulting in high cost for downstream processing [11]. The limiting step in microbial TTMP production is physiology [11], and attention has been focused on breeding of strains [3, 4, 18, 23], optimization of medium formulation and culture conditions [3, 10, 18, 19, 21, 23], and identification of precursors [1, 3, 9, 10]. It was postulated that one mole of TTMP was condensed from two moles of 3-hydroxy-2-butanone (HB) and two moles of ammonia [11].

Culture pH is one of the most important factors affecting cell growth and product formation rates. Larroche et al. [10] observed that TTMP production remained almost unchanged when controlling culture pH at 7.0 compared with that of uncontrolled process, while Xiao et al. [18] found that stable neutral pH was necessary for better TTMP accumulation. To date, except for the observations above, there have been few reports about effects of culture pH on cell growth, precursor HB accumulation, and corresponding TTMP production in batch fermentation using *Bacillus* sp. strains.

Previously, a *Bacillus* sp. strain was isolated from a Chinese *Maotai*-flavor Daqu using an endogenous precursor strategy [22]. The process of TTMP fermentation by the *Bacillus* sp. strain could be divided into two phases, including the cell growth and precursor HB accumulation phase and the TTMP production phase. Therefore, it is necessary to investigate the optimal pH profile to maximize final TTMP concentration. The objective of this work is to elucidate the function of culture pH on cell growth, precursor HB accumulation, and TTMP formation in batch fermentation by *Bacillus* sp., and an optimum pH control strategy was accordingly developed to enhance TTMP production.

Materials and methods

Microorganism and medium

The microorganism used in this study was a *Bacillus subtilis* isolated from a high-temperature *Maotai*-flavor Daqu [22], and was deposited in the China Center for Type Culture Collection (CCTCC M 208157).

The seed medium contained 80 g l^{-1} glucose, 10 g l^{-1} peptone, and 10 g l^{-1} yeast extract, with initial pH 7.2. The flask medium contained 100 g l^{-1} glucose, 25 g l^{-1} yeast extract, and 30 g l^{-1} diammonium phosphate. The batch fermentation medium used in the fermentor contained 100 g l^{-1} glucose, 25 g l^{-1} yeast, and 30 g l^{-1} diammonium phosphate. The initial pH of medium was adjusted with 10 M HCl or 10 M NaOH. Glucose was sterilized separately. All media were autoclaved at 121°C for 20 min.

Shake flask fermentation

Seed culture was prepared by transferring a loopful of cells from a slant culture into 20 ml sterile seed medium in 100-ml Erlenmeyer flask and incubated with shaking (200 rpm) at 37°C for 20 h. The seed culture ($2-3 \times 10^8$ cells/ml) was then inoculated (5%, v/v) into 250-ml Erlenmeyer flasks containing 50 ml flask medium, and incubated with shaking (200 rpm) at 37°C for 144 h. All experiments were performed in duplicate and the mean determined.

Bioreactor fermentation in 7.5-l fermentor

Bioreactor fermentation was carried out in a 7.5-l fermentor (BioFlo 110; New Brunswick Scientific, Edison, NJ). Working volume was controlled at 5 l. *Bacillus subtilis* CCTCC M 208157 was precultured in flask medium (50 ml medium in 250-ml Erlenmeyer flask) with shaking (200 rpm) at 37°C for 20 h before being inoculated (5%, v/v) into the fermentor. The fermentor was run at 37°C with stirring (500 rpm), air was supplied at flow rate of 1.0 vvm, and pH was controlled at a set value automatically with 28% (w/w) ammonia solution or 10 M HCl during fermentation. All experiments were performed in duplicate and the mean determined.

Analytical methods

Samples were withdrawn every 12 h from the flask or fermentor to analysis cell growth, residual sugar, and HB and TTMP content. Each sample was split into two aliquots. One of them was centrifuged (10,000 × *g*, 10 min), and the precipitate was collected, washed twice with distilled water, and dried at 105°C to constant weight to determine the biomass of the culture according to Hu et al. [7]. The supernatant was used to determine total sugar using 3,5-dinitrosalicylate method as described by Miller [14]. Another aliquot was diluted to appropriate folds with phosphate buffer (0.5 M, pH 7.0) to assay for precursor HB and TTMP according to the literature [22].

Results and discussion

Effects of initial pH on TTMP fermentation in flasks

Figure 1 shows the process of TTMP fermentation with initial pH 5.0 and 7.0 in flask experiments. The flask medium contained a sufficient amount of phosphate to stabilize culture pH at certain range. It was clear that pH played a crucial role in TTMP biosynthesis. Cultivation with initial pH 5.0 gave low final TTMP concentration (0.22 g l^{-1}), while higher concentration of 4.12 g l^{-1}

TTMP was obtained in the cultivation with initial pH 7.0 after 144 h of fermentation.

HB is an important physiological metabolic product derived from pyruvate, and its function as the precursor of TTMP has been verified in several studies [3, 9, 18, 22]. It was observed that precursor HB was excreted simultaneously with cell growth and accumulated continuously by *Bacillus subtilis* CCTCC M 208157 in early stationary phase. More accumulation of endogenous precursor HB was found in lower pH condition, which could be explained by the increased amount of biomass and the activated acetolactate synthase (ALS, also called "pH 6 enzyme," responsible for acetolactate formation in the acetoin/2,3-butanediol pathway) in acidic conditions [12]. Although the amount of biomass in cultivation with initial pH 5.0 was higher (Fig. 1c), a considerably prolonged lag phase of cell growth and low precursor HB accumulation rate in early cultivation period were observed.

The effects of varying initial pH ranging from 5.0 to 7.5 on TTMP synthesis, precursor HB accumulation, and cell growth in flask experiments were investigated (Fig. 2). Optimal initial pH for cell growth and precursor HB accumulation was found to be 5.5 with 12.5 g l^{-1} biomass and 36.7 g l^{-1} maximum HB, while optimal pH for TTMP formation occurred at 7.5 with TTMP yield of 4.71 g l^{-1}. The results suggested that cell propagation and HB accumulation were favored in acidic conditions, while TTMP was synthesized more rapidly in conditions with initial pH towards neutrality. Similar results appeared in the previous work, in which it was reported that total concentration of HB and 2,3-butanediol increased obviously with decrease of pH from 6.4 to 5.5 when using *Enterobacter aerogenes* DSM 30053 in a membrane bioreactor with cell recycling [20], and a stable neutral pH condition was found to be necessary for better TTMP production by *Bacillus* sp. RX3-17 [18]. The above results suggest that culture pH plays a vital role in cell growth, precursor HB accumulation, and TTMP formation. Therefore, it was essential to elucidate the function of culture pH on TTMP batch fermentation in controlled pH processes.

Time course of batch TTMP fermentation in fermentor

Bioreactor fermentations were performed in a 7.5-l fermentor under various controlled pH ranging from 5.5 to 7.0 to examine the time profiles of cell growth, precursor HB accumulation, and TTMP production as well as sugar utilization (Fig. 3). At pH lower than 5.5, TTMP concentration was very low, while in conditions with pH higher than 7.0, precursor HB accumulation decreased sharply. Thus, the time-course data of these fermentations and the related kinetic parameters are not presented.

The profiles of precursor HB accumulation in various controlled pH cultivations exhibited basically the same saddle shape (Fig. 3), while the peak value of precursor HB decreased monotonously with increasing pH. In contrast, TTMP was synthesized more rapidly with increasing pH, and higher TTMP production was observed in cultivations with culture pH controlled towards neutrality. The results demonstrated that the effect of culture pH on synthesis of TTMP was significant.

The sharp decrease of precursor HB was found in late cultivation as viable cell numbers decreased in the broth (data not shown), which is in accordance with our previous

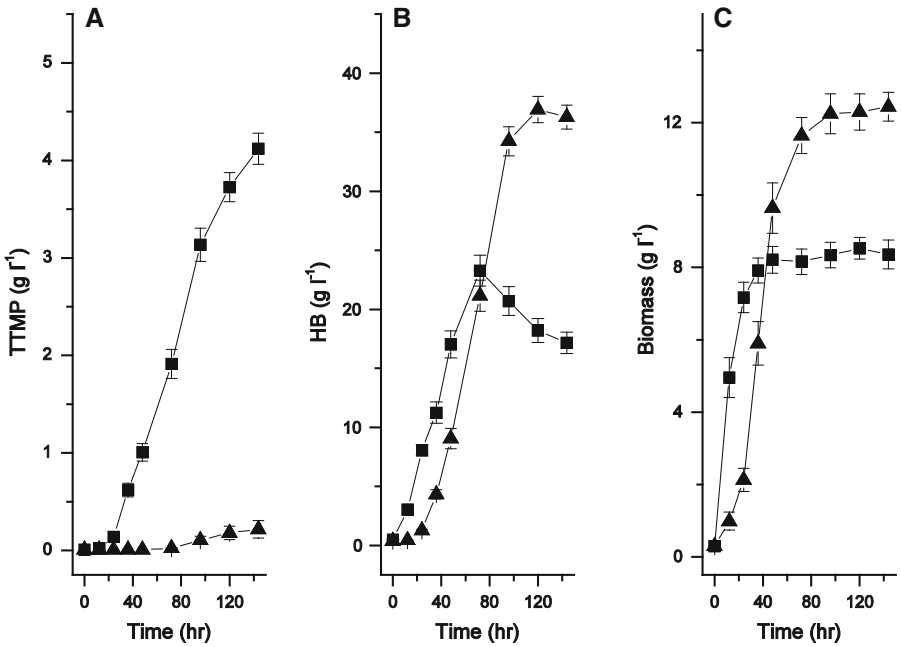

Fig. 1 Time course of TTMP (**a**), precursor HB (**b**), and biomass (**c**) during cultivation of *B. subtilis* CCTCC M 208157 in 250-ml flasks with initial pH 5.0 (*filled triangle*) and 7.0 (*filled square*), respectively

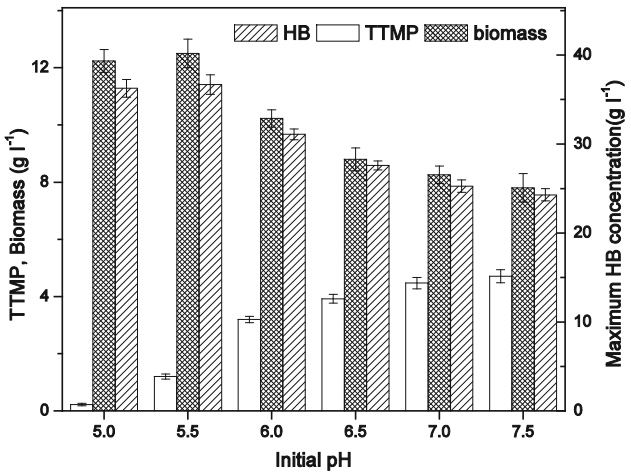

Fig. 2 Effects of various initial pH on biomass (▩), precursor HB (▨), and TTMP content (□) after 144 h of cultivation in flask experiments

report [22]. Despite metabolic flux to TTMP formation, further reduction of precursor HB was observed after 48 h of cultivation. The reason could be the formation of byproduct from the reaction of precursor HB and ammonia [17], and also the physiological function of HB, which is an energy-storing material derived from pyruvate. Due to glucose depletion combined with adverse conditions caused by increasing amount of TTMP and probably the existence of other toxic metabolites in late cultivation environment, HB was consumed as the successive energy and carbon source to keep cells active [5].

Increases of biomass and precursor HB were obtained in fermentation when controlled culture pH was lower. No significant lag phase of cell growth was observed in 7.5-l fermentor, probably due to elevation of the oxygen supply rate in the fermentor environment. The residue sugar kept declining rapidly in cell propagation and precursor HB accumulation periods, and declined slowly as precursor HB began to decrease.

Effect of culture pH on kinetics of bioreactor fermentation

Fermentation kinetic parameters of bioreactor fermentation under various controlled pH processes are listed in Table 1. The yield of TTMP from maximum precursor HB ($Y_{TTMP/HB}$) was introduced to interpret the conversion ability of precursor to desired TTMP product in fermentative environment. Although the peak value of precursor HB (P_{HBmax}) increased monotonically from 19.95 to 30.19 g l^{-1} with decrease in culture pH from 7.0 to 5.5, higher TTMP concentration was found in higher pH

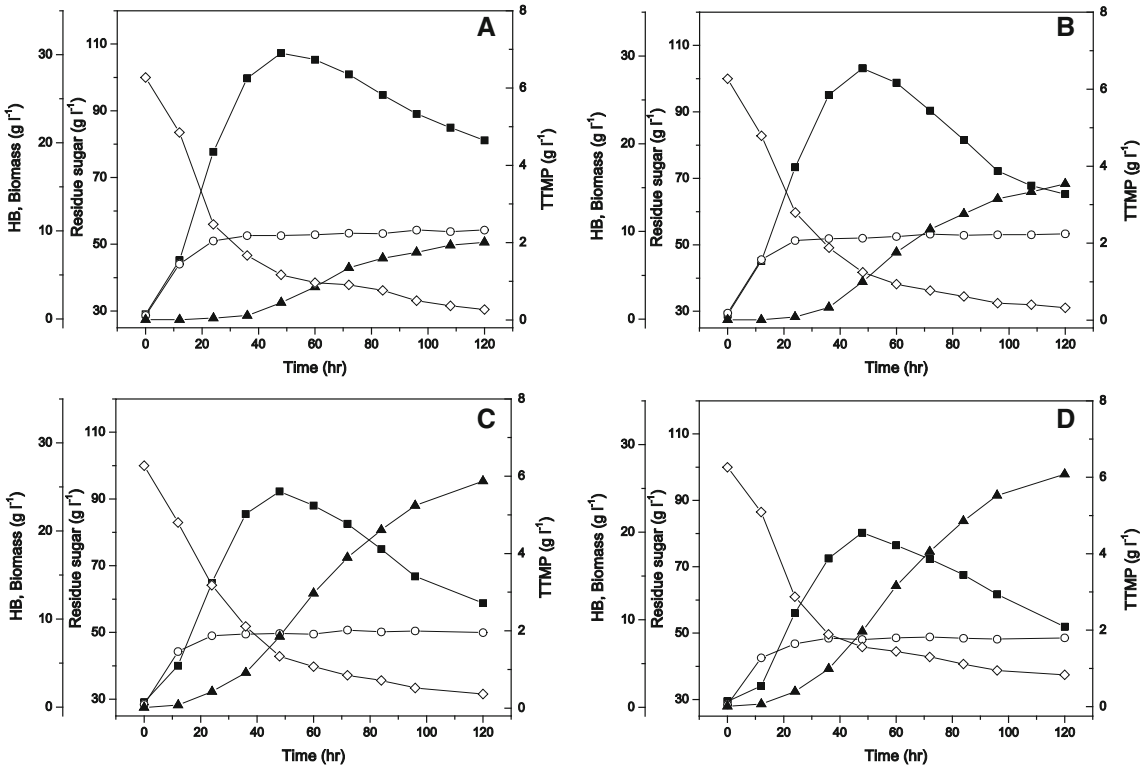

Fig. 3 Time course of batch fermentation in 7.5-l fermentor under various controlled pH processes: **a** pH 5.5, **b** pH 6.0, **c** pH 6.5, and **d** pH 7.0, respectively, for TTMP (*filled triangle*), HB (*filled square*), glucose (*open diamond*), and biomass (*open circle*). Data are means of duplicates. Standard errors were less than 5.0% of the means

Table 1 Kinetic parameters of batch TTMP fermentation under various controlled pH processes in 7.5-l fermentor

pH controlled	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH shifted[a]
X_{max} (g l^{-1})	9.46	9.23	8.45	7.89	9.49
P_{TTMP} (g l^{-1})	2.01	3.54	5.97	6.08	7.43
P_{HBmax} (g l^{-1})	30.19	28.61	25.31	19.95	30.11
Q_x (g l^{-1} h^{-1})	0.833	0.828	0.786	0.707	0.837
Q_{TTMP} (g l^{-1} h^{-1})	0.038	0.061	0.088	0.095	0.180
Q_{HB} (g l^{-1} h^{-1})	1.118	1.108	0.879	0.56	1.116
$Y_{x/s}$ (g g^{-1})	0.14	0.13	0.12	0.13	0.12
$Y_{HB/x}$ (g g^{-1})	3.19	3.10	3.01	2.53	3.17
$Y_{TTMP/HB}$ (g g^{-1})	0.07	0.13	0.23	0.30	0.24

Data are means of duplicates. Standard errors were less than 5.0% of the means

[a] Culture pH was controlled at pH 5.5 within the first 48 h and then shifted to pH 7.0 until the end of the fermentation

controlled process. The discrepancy of pH effects on precursor HB and TTMP accumulation resulted in significant dissimilarity of $Y_{TTMP/HB}$. A higher $Y_{TTMP/HB}$ value of 0.30 g g^{-1} was obtained when culture pH was controlled at pH 7.0, compared with that of cultivations with other pH towards weak acidity. Compared with the theoretical yield of TTMP from precursor HB (0.77 g TTMP g HB^{-1}), the yield obtained in this work was considerably higher, while the yield of exogenously added precursor approach for enhanced TTMP production was only 0.0008 g g^{-1} [10].

The influence of culture pH on cell growth of *B. subtilis* in the controlled pH fermentation bioprocess was significant, as indicated in Table 1. When culture pH was controlled from 7.0 to 5.5, biomass (X_{max}) increased monotonically from 7.89 to 9.46 g l^{-1}, and the optimal growth rate (Q_x) and cell yield ($Y_{x/s}$), occurring at pH 5.5, were 0.833 g l^{-1} h^{-1} and 0.136 g g^{-1}, respectively. Similarly, the maximum precursor HB concentration (P_{HBmax}) showed an optimal value of 30.19 g l^{-1} at pH 5.5, and decreased by 33.9% at pH 7.0. The optimal precursor HB formation rate (Q_{HB}) and yield ($Y_{HB/x}$), occurring at pH 5.5, were 1.118 g l^{-1} h^{-1} and 3.19 g g^{-1}, respectively. The results obtained above suggested that *B. subtilis* cells were more vegetative in weak acidic conditions, and more vigorous in supporting precursor HB accumulation probably due to activated acetolactate synthase [5].

In contrast to the impact of culture pH on cell growth and precursor HB accumulation, TTMP was synthesized more efficiently in neutral conditions. Figure 4 shows profiles of the specific TTMP formation rate (q_{TTMP}) and specific precursor HB accumulation rate (q_{HB}) under various controlled pH bioprocesses. Although the profiles of q_{TTMP} and q_{HB} exhibited similar tendencies, higher values of q_{HB} were found in acidic cultivations, while in neutral conditions the values of q_{TTMP} were significantly enhanced. It is thus presumed that precursor HB and TTMP accumulation in fermentative system by *B. subtilis* CCTCC M 208157 were separately regulated. To improve the efficiency of TTMP production in bioreactor fermentation, an acidic condition was preferred to ensure high accumulation of precursor HB in early stage of cultivation, and a neutral environment was then required for enhanced TTMP synthesis after maximal concentration of precursor HB was reached.

pH-Shifted bioreactor fermentation process for enhanced TTMP production

Based on the discrepancy of optimal pH for precursor HB accumulation and TTMP formation, it was favorable to use a pH-shifted controlled process instead of a constant-pH controlled process. An optimal pH-shifted controlled strategy was developed to optimize TTMP production as follows: firstly, culture pH was controlled at pH 5.5 to allow cell propagation and sufficient precursor HB accumulation during the first 48 h of cultivation, and then pH was shifted to 7.0 to further promote TTMP synthesis in later cultivation. The fermentation process and kinetic parameters are demonstrated in Fig. 5 and Table 1. As expected, TTMP production (7.43 g l^{-1}) in the pH-shifted

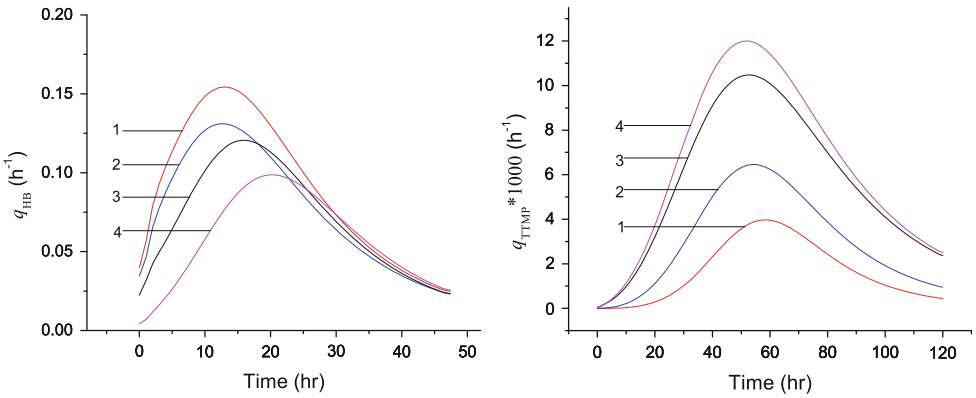

Fig. 4 Time course of specific TTMP formation rate (q_{TTMP}) and specific precursor HB accumulation rate (q_{HB}) under various controlled pH processes: (1) pH 5.5, (2) pH 6.0, (3) pH 6.5, and (4) pH 7.0

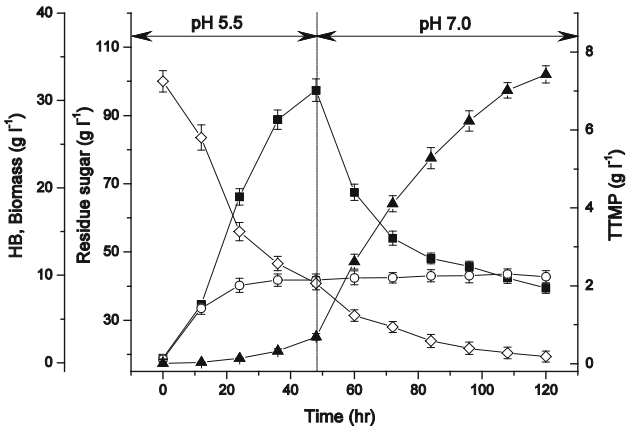

Fig. 5 Batch fermentation with culture pH shifted from 5.5 to 7.0 after 48 h fermentation in 7.5-l fermenter by *Bacillus subtilis* CCTCC M 208157: TTMP (*filled triangle*), HB (*filled square*), glucose (*open diamond*), and biomass (*open circle*)

controlled bioreactor fermentation process was further enhanced by 22.2% compared with that of fermentation with pH controlled at 7.0. Biomass and precursor HB accumulation were basically the same as that of the pH 5.5 controlled process, while the TTMP formation rate (Q_{TTMP}) and yield from precursor HB ($Y_{TTMP/HB}$) of the pH-shifted controlled fermentation process were significantly enhanced to 0.180 g l^{-1} h^{-1} and 0.25 g g^{-1}, respectively. The results above demonstrate the effectiveness of the pH-shifted controlled strategy in supporting enhanced TTMP production with better cell propagation and high accumulation of precursor HB.

Conclusions

This work demonstrated a critical influence of culture pH on microbial TTMP production in shake flask and bioreactor fermentation. Weak acidic conditions were found to favor cell growth and precursor HB accumulation, while TTMP could be synthesized more efficiently in conditions with culture pH towards neutrality. A pH-shifted controlled fermentation process was accordingly developed and demonstrated to be effective for enhanced TTMP production. Final TTMP concentration of 7.43 g l^{-1} was obtained, being 22.2% greater than that of fermentation with constant pH controlled at 7.0. By fine-tuning of the pH-shifted strategy, there may be scope for further enhancement of TTMP production.

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