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Research Article

Effect of nitrogen, carbon sources and agitation speed on acetoin production of *Bacillus subtilis* SF4-3

Yanjun Tian [a], Yixiao Fan [b], Jianjun Liu [b], Xiangying Zhao [b], Wei Chen [a,*]

[a] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, PR China
[b] Key Laboratory of Food and Fermentation Engineering of Shandong Province, Shandong Food Ferment Industry Research & Design Institute, Jinan 250013, PR China

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ABSTRACT

Background: Currently, microbial fermentation method has become the research hotspot for acetoin production. In our previous work, an acetoin-producing strain, *Bacillus subtilis* SF4-3, was isolated from Japanese traditional fermented food natto. However, its conversion of glucose to acetoin was relatively low. In order to achieve a high-efficient accumulation of acetoin in *B. subtilis* SF4-3, main medium components and fermentation conditions were evaluated in this work.
Results: The by-products analysis showed that there existed reversible transformation between acetoin and 2,3-butanediol that was strictly responsible for acetoin production in *B. subtilis* SF4-3. The carbon sources, nitrogen sources and agitation speed were determined to play crucial role in the acetoin production. The optimal media (glucose·H_2O 150 g/L, yeast extract 10 g/L, corn steep dry 5 g/L, urea 2 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.5 g/L) were obtained. Furthermore, the low agitation speed of 300 r/min was found to be beneficial to the reversible transformation of 2,3-butanediol for acetoin production in *B. subtilis* SF4-3. Eventually, 48.9 g/L of acetoin and 5.5 g/L of 2,3-butanediol were obtained in a 5-L fermenter, and the specific production of acetoin was 39.12% (g/g), which accounted for 79.90% of the theoretical conversion.
Conclusions: The results indicated acetoin production of *B. subtilis* SF4-3 was closely related to the medium components and dissolved oxygen concentrations. It also provided a method for acetoin production via the reversible transformation of acetoin and 2,3-butanediol.

1. Introduction

Acetoin is a kind of pale yellow or colorless liquid with a typical cream, fat, butter-like aroma. It is one frequently used spice additive all over the world, which is always used to make butter, dairy products, nuts, flavors and other flavor substances. In addition, acetoin is also an important platform compound, which has been listed as one of 30 kinds of platform compounds given priority exploitation in 2004 by the United States Department of Energy. Its derived products can be applied in the pharmaceutical industry, chemical industry, paint industry, information technology industry and so on. Currently, the production of acetoin in the industry is still based on chemical synthesis using non-renewable petrochemical resources as a raw material. With the depletion of global petrochemical resource, deterioration of environment problems and improvement of people's demand for green products, traditional chemical synthesis process with high pollution, high energy will be gradually eliminated, while production of acetoin by microbial fermentation has advantages of pure natural product, rich source of raw materials, mild conditions, environment friendly, etc. Now, this has become the research hotpot for acetoin production.

Many microorganisms are capable of producing acetoin in nature, which is an intermediate product of carbon overflow metabolism, and whose secretion plays a vital role in the cell growth, including avoiding acidification of internal environment, regulating the balance of reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide (NAD) ratio, and stored as carbon source [1]. In the past years, the metabolic pathways of acetoin in most microorganisms has been studied intensively and the well-known one is as follows [2]: Firstly, pyruvate is synthesized by the glycolytic pathway using sugar feedstocks in vivo, then two molecules of pyruvate are transformed into an α-acetolactate and carbon dioxide under the catalysis of α-acetolactate synthase (ALS), and then acetolactate decarboxylase (ALDC) acting on α-acetolactate generates a molecule of acetoin. Furthermore, acetoin can be reduced to 2,3-butanediol under the reversible 2,3-butanediol dehydrogenase (BDH) (Fig. 1). In the early studies, acetoin is primarily mentioned as one by-product of 2,3-butanediol production via microbial synthesis, and its production is generally less than 20.0 g/L, such as in *Klebsiella pneumoniae* [3,4], *Klebsiella oxytoca* [5,6,7,8], *Serratia marcescens* [9],

* Corresponding author.
E-mail address: chenwei66@jiangnan.edu.cn (W. Chen).
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Fig. 1. Metabolic pathway of acetoin and 2,3-butanediol in bacteria.

Bacillus amyloliquefaciens [10,11], *Paenibacillus polymyxa* [12,13], etc. Recently, acetoin has been paid more attention and reports relative to its research as the target product gradually increase, a number of potential industrial production strains has been isolated, but 2,3-butanediol was still a major by-product of its production [14,15,16,17,18,19,20,21]. Given that acetoin and 2,3-butanediol are always generated concomitantly in the microorganisms, the optimization aiming at enhancing acetoin production was still focused on reducing 2,3-butanediol production for obtaining high-yield acetoin strains. Sun et al. [22] employed statistical optimization method and two-stage speed control strategy to improve acetoin production; acetoin titer in *S. marcescens* H32 was increased to 60.5 g/L along with 20.0 g/L of 2,3-butanediol. Zhang et al. [23] isolated an acetoin-producing *P. polymyxa* CS107, after the medium optimization and dissolved oxygen control strategy, acetoin production could reach 55.3 g/L with only 10.0 g/L of 2,3-butanediol. However, there are a few literatures about its production based on the reversible transformation between acetoin and 2,3-butanediol in the microorganisms.

In our previous work, an acetoin-producing *B. subtilis* SF4-3 has been isolated from Japanese fermented food natto, and a phenomenon that concentration of acetoin continued to increase after exhaust of glucose in the medium was found [24,25]. In this study, a 5-L fermentation experiment was carried out, and the reversible transformation of 2,3-butanediol and acetoin was discovered to be responsible for the increase of acetoin concentration at the late stage of fermentation. Then, the optimization experiments were conducted and found that carbon sources, nitrogen sources and agitation speed played an important role in the transformation of acetoin and 2,3-butanediol. Finally, a suitable operation process was established for acetoin production of *B. subtilis* SF4-3 in a 5-L fermenter.

2. Materials and methods

2.1. Bacterial strain

B. subtilis SF4-3 was isolated from the Japanese traditional food "natto" and reserved in our laboratory. It was maintained on agar slants containing the following media: glucose·H_2O 10 g/L, yeast extract 5 g/L, peptone 10 g/L, sodium chloride 10 g/L, and agar 20 g/L at pH 7.0. The slants were sub-cultured and incubated at 37°C for 24 h, and then stored at 4°C for experimental use.

2.2. Cultivation of B. subtilis SF4-3 in shake flasks

The inoculums were prepared by transferring one full loop of cells from the freshly prepared ager slants to an 500-mL shake flask containing 50-mL seed medium (glucose·H_2O 50 g/L, yeast extract 5 g/L, corn steep dry 10 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, pH 7.00) and incubated at 37°C on a rotary shaker at 180 r/min for 8–12 h. Subsequently, the inoculums (5%, v/v) were inoculated into 500-mL shake flasks containing 50-mL fermentation medium (glucose·H_2O 150 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.5 g/L, pH 7.00). In order to investigate the effect of nitrogen sources on acetoin fermentation of strain SF4-3, different concentrations of organic nitrogen sources, yeast extract (5, 10, 20, 30, 40 g/L), corn steep dry (5, 10, 20, 30, 40 g/L) and inorganic nitrogen sources (urea 1.50 g/L, $(NH_4)_2HPO_4$ 3.30 g/L, KNO_3 5.05 g/L) were firstly added in the fermentation medium, respectively. The samples were taken every 24 h during the fermentation process. Then, the cell free supernatants were harvested by centrifugation at 5000 r/min for 15 min and then transferred to new vials for acetoin determination.

2.3. Batch fermentation of B. subtilis SF4-3 in 5-L fermenter

The batch fermentation was carried out in a 5-L stirred fermenter (Shanghai Baoxing Bioengineering Equipment Co., Shanghai, China) with an initial broth volume of 3.5 L fermentation medium. Firstly, the pre-culture medium was prepared as above 2.2. After incubation at 37°C for 8–12 h on a rotary shaker (180 r/min), the pre-culture was inoculated (5%, v/v) into the optimized medium with an initial pH value of 7.00. The cultivation was carried out at 37°C and pH was not controlled during the fermentation process. In order to explore the optimal dissolved oxygen control strategy, the airflow was kept at 0.5 vvm and different agitation speed of 300, 400, 500, and 500–300 r/min were independently investigated during the batch fermentation.

2.4. Analytical methods

To monitor the cell growth at different incubation times, the fermentation broths were diluted to appropriate folds, and the biomass concentrations were determined by the optical density (OD) at 600 nm using a spectrophotometer. Then fermentation broths were centrifuged at 5000 r/min for 15 min and the supernatants were diluted to 50–200 folds for residual glucose determinations using a biological sensing analyzer equipped with a glucoseoxidase electrode (SBA-40D, Institute of Biology, Shandong Academy of Sciences, Shandong, China). Acetoin and 2,3-butanediol in the supernatants (1–5 folds of dilution in advance) were extracted by ethyl acetate with the addition of n-butanol as the internal standard and then was measured by a gas chromatography (GC) system (Agilent GC7890B, California, USA) equipped with a flame ionization detector and a DB-WAX capillary column (30 m × 0.32 mmi.d × 0.25 μmd.f, Agilent Technologies, California, USA). The operation conditions were as follows: the high purity nitrogen was used as carrier gas at a flow rate of 1.5 mL/min, the temperatures of injector and detector were both 220°C, and the column oven was kept at 50°C for 5 min, then programmed to 240°C with a stepwise increase of 30°C/min and maintain at 240°C for 3 min. The injection volume was 1 μL.

3. Results and discussion

3.1. Reversible transformation of acetoin and 2,3-butanediol in B. subtilis SF4-3

In our laboratory, an acetoin-producing *B. subtilis* 4-3 has been isolated from the Japanese fermented food natto. In order to further explore the key factors which affected the conversion of glucose to acetoin, the batch fermentation was carried out in a 5-L fermenter for monitoring the fermentation process and by-product formation.

As shown in Fig. 2, after the start of fermentation, the dissolved oxygen dramatically declined to 0% at 12 h and maintained a relatively low level (<0%) until its slow rise suddenly occurred after 39 h. The pH value appeared slow rise (0–39 h) and decline (39–48 h), then its sharp increase occurred after the glucose in the medium was exhausted. As for the cell mass, it could be seen from the changes of OD value that it entered into the steady stage at

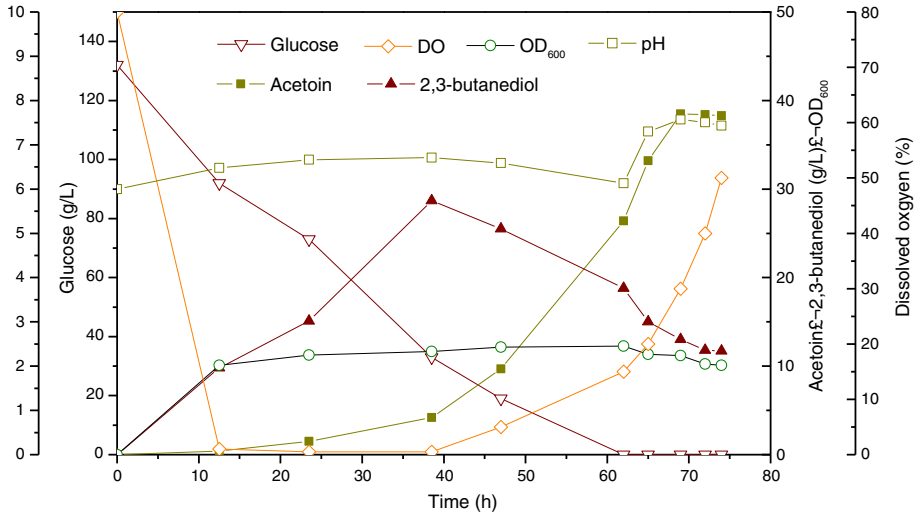

Fig. 2. Time profiles of batch fermentation of acetoin by *B. subtilis* SF4-3. The initial glucose concentration was 150 g/L and the initial pH value was 6.0. The nitrogen sources were composed of YE (10 g/L) and CSD (15 g/L). The dissolved oxygen was connected with the agitation by increasing the agitation speed until 500 r/min. The pH value was not adjusted during the fermentation process.

24 h and kept a stable value afterwards. The product analysis indicated that acetoin concentration appeared a slow increase in the first 24 h. However, after 24 h, its concentration started to increase sharply. However, it was worthwhile pointing out that its concentrations continued to increase for a while after the carbon source in the medium was exhausted. However, the by-product analysis indicated that no remarkable amount of acetate, ethanol, lactate and succinate were formed in the fermentation broth. According to the literatures, acetoin and 2,3-butanediol were always produced concomitantly in most microorganisms. The analytical results indicated that the concentration of 2,3-butanediol began to increase drastically at the start of fermentation until its concentration reached 41.7 g/L at 39 h. Then, its concentration began to decline sharply after 39 h accompanied by an increase of acetoin production. Taking the metabolic pathways of acetoin and 2,3-butanediol in bacteria into account, acetoin and 2,3-butanediol can been transformed into each other directly [26,27,28]. In this context, there should be a transformation of acetoin and 2,3-butanediol in *B. subtilis* SF4-3. Initially, the produced acetoin was mainly transformed to 2,3-butanediol, and afterwards, the majority of 2,3-butanediol was conversely transformed to acetoin. Therefore, when the carbon source in the medium was consumed completely, the reason that concentration of acetoin appeared further enhancement to a degree was due to the reversible transformation of 2,3-butanediol. In this process, one molecule of NADH would be regenerated via the BDH for maintaining cell growth.

3.2. Effect of organic nitrogen sources on the fermentation of B. subtilis SF4-3

In order to achieve maximum transformation of 2,3-butanediol and acetoin production, the effect of nitrogen sources on the transformation was firstly investigated in shake flasks. According to the recent reports, the complex organic nitrogen source would be beneficial for the production of acetoin and 2,3-butanediol [29]. Therefore, *B. subtilis* SF4-3 was cultivated in the simple glucose containing medium supplemented with various concentrations of yeast extract (YE) and corn steep dry (CSD), respectively. The results indicated that synthesis of acetoin and 2,3-butanediol were significantly promoted by their increasing concentrations (Fig. 3).

When using YE as the sole nitrogen source, the formation of products were significantly different under different concentrations of YE addition. At the start of 24 h, the high amount of YE addition resulted in more 2,3-butandediol production, while it exerted some negative effect on the acetoin production (Fig. 3a). But when it proceeded to 72 h, the reversible transformation of 2,3-butanediol to acetoin happened to the high YE addition medium (20 g/L, 30 g/L, or 40 g/L), and the majority of 2,3-butanediol was conversely transformed to acetoin (Fig. 3b). By contrast, the fermentation corresponding to a small addition (5 g/L or 10 g/L) produced little acetoin and 2,3-butanediol at 24 h and 72 h, and no reversible transformation of 2,3-butanediol to acetoin took place. A similar effect on acetoin production was observed when CSD was used instead of YE (Fig. 3c and d). Compared with YE, the fermentations with low concentrations of CSD addition (5 g/L or 10 g/L) were less productive due to its low cell mass. While, high amounts of CSD addition were also beneficial to 2,3-butanediol production at the early stage, which was almost transformed to acetoin at the end of fermentation. But, when using YE as nitrogen source, the reversible transformation occurred more early than that with CSD at the same addition. In summary, YEs were chosen as the preferential nitrogen source for acetoin production of *B. subtilis* SF4-3.

These above results demonstrated that rich fermentation medium with adequate nitrogen source (YE or CSD) played an important role in the acetoin production of *B. subtilis* SF4-3. Under the conditions of low nutritional medium, *B. subtilis* SF4-3 consumed the carbon source in a relatively slow speed, and consequently, merely a small quantity of acetoin and 2,3-butanediol were formed. In contrast to the fermentation with low nutritional medium, the cells in the rich nutritional medium multiplied rapidly, and 2,3-butanediol was largely synthesized firstly along with little accumulation of acetoin. When the fermentation came into later phase, the produced 2,3-butanediol was conversely transformed to acetoin for maintaining its metabolism. The results indicated that acetoin production of *B. subtilis* SF4-3 was highly associated with cell growth and an appropriate cell growth would be advantageous for acetoin production [18]. Additionally, it was worthwhile noting that the viscosity of fermentation broth began to increase along with enhancement of nitrogen source addition. As far as I am concerned, the phenomenon would lead to the formation of micro-anaerobic environment and thereafter affected the cell growth and its metabolism to some extent. This also may be one main reason that could explain the substantial accumulation of 2,3-butanediol in the early phase of fermentation.

As mentioned in above, high YE addition was more superior to acetoin production of *B. subtilis* SF4-3 compared with the CSD addition. But, taking the high cost of YE into account, the combinations of YE and

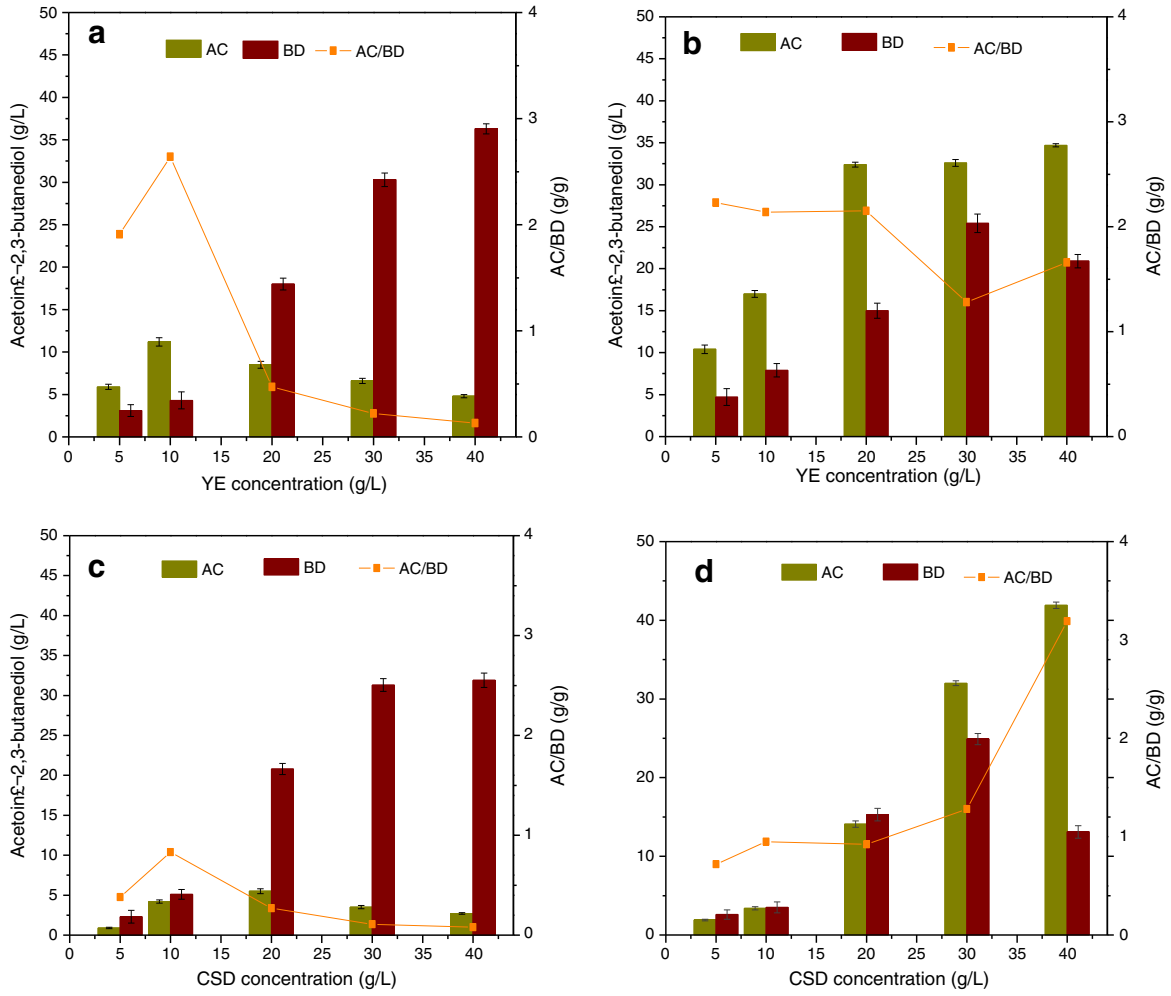

Fig. 3. Effect of initial YE or CSD concentrations on acetoin fermentation by *B. subtilis* SF4-3 during the fermentation process in shake flask. The initial glucose concentration was 150 g/L and the initial pH value was adjusted to 7.00. (a) Culture time: 24 h; (b) culture time: 72 h; (c) culture time: 24 h; (d) culture time: 72 h.

CSD on its acetoin production was investigated below (Table 1). The maximum acetoin production (47.8 g/L) was achieved under the combination of 20 g/L YE and 15 g/L CSD. The small amount of combination of YE and CSD exhibited little cell mass and slower glucose consumption, and little acetoin and 2,3-butanediol were formed. This obtained results further illustrated that the rich nutritional medium is beneficial to reversible transformation of 2,3-butanediol for acetoin production of *B. subtilis* SF4-3, which is consistent with the result using the sole YE or CSD.

Table 1
Effect of different concentrations of YE and CSD addition on acetoin fermentation by *B. subtilis* SF4-3. The initial glucose concentration was 150 g/L. The nitrogen sources were added as follows. The initial pH value was adjusted to 7.00 and the incubation time was 72 h.

No.	YE (g/L)	CSD (g/L)	OD_{600}	RG[a] (g/L)	AC (g/L)	BD (g/L)	AC/BD
1	20	5	9.31 ± 0.12	0	39.3 ± 0.5	20.6 ± 0.8	1.91
2	20	10	9.45 ± 0.13	0	43.8 ± 0.6	11.5 ± 0.9	3.81
3	20	15	9.57 ± 0.15	0	47.8 ± 0.6	7.2 ± 0.7	6.64
4	15	5	7.12 ± 0.10	11.0 ± 1.5	34.2 ± 0.2	16.2 ± 0.8	2.11
5	15	10	7.66 ± 0.09	0	38.3 ± 0.2	28.0 ± 0.6	1.37
6	15	15	9.11 ± 0.11	0	43.2 ± 0.3	13.4 ± 0.9	3.22
7	10	5	6.84 ± 0.13	39.5 ± 1.2	20.6 ± 0.4	14.6 ± 1.0	1.41
8	10	10	7.99 ± 0.13	15.5 ± 1.3	30.5 ± 0.5	20.3 ± 0.6	1.50
9	10	15	8.84 ± 0.14	0	32.0 ± 0.4	31.0 ± 0.8	1.03
10	5	5	7.60 ± 0.13	78.3 ± 1.4	7.7 ± 0.3	6.5 ± 0.7	1.18
11	5	10	7.98 ± 0.12	55.4 ± 1.2	11.8 ± 0.4	7.6 ± 0.7	1.55
12	5	15	8.20 ± 0.1	15.8 ± 1.0	26.0 ± 0.4	19.5 ± 0.6	1.33

[a] RG, residual glucose concentrations in the medium.

3.3. Effect of inorganic nitrogen sources on the fermentation of *B. subtilis* SF4-3

Although the above experiment elucidated that acetoin production of *B. subtilis* SF4-3 could greatly affected by the rich nutritional medium, it should be noted that rich nutritional medium also led to high viscosity of fermentation broth that would have an adverse impact on its downstream process. This operational instability precluded from the use of high contents of YE or CSD as the nitrogen source in spite of its much higher acetoin production. Thus, based on the lower nutritional medium (YE 5 g/L and CSD 5 g/L), the inorganic nitrogen sources with the same NH_4^+ concentration of 50 mM (e.g. urea 1.5 g/L, diammonium hydrogen phosphate 3.3 g/L or potassium nitrate 5.05 g/L) were independently added to investigate their effects on the acetoin production of *B. subtilis* SF4-3. Fig. 4 described the time courses of optimal density, residual glucose, pH, acetoin and 2,3-butanediol during the fermentation process, respectively.

When using urea as the substitute of nitrogen source, its pH value dropped to 5.80 (24 h) and maintained a constant level until carbon source in the fermentation broth was exhausted (96 h). The optical density rapidly increased to 11.28 at 24 h and appeared a slight decline after 48 h of cultivation. It was also noted that concentration of 2,3-butanediol declined slightly at 24 h, and concentration of acetoin started to increase significantly. Finally, the maximum acetoin

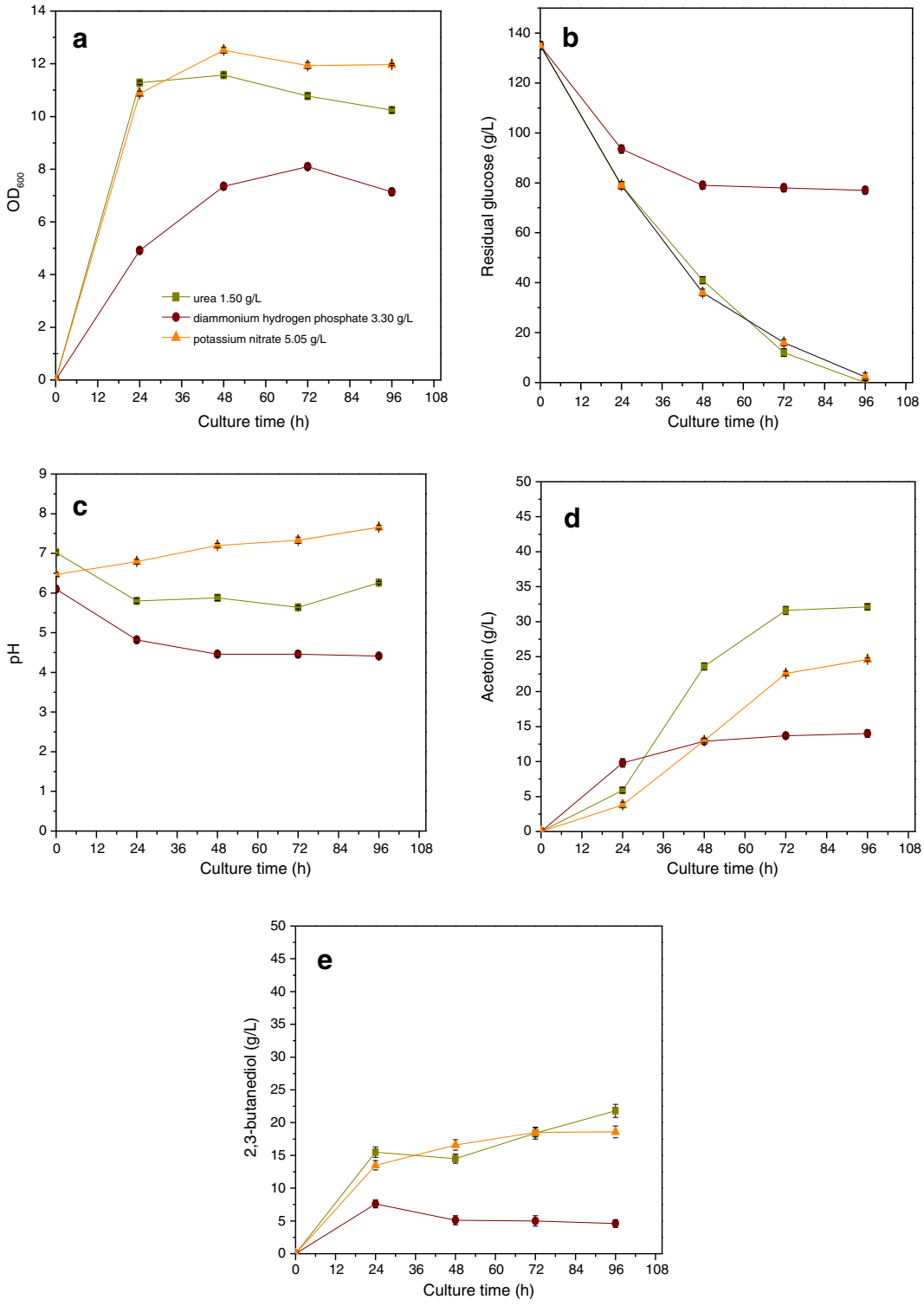

Fig. 4. Influence of added urea, diammonium hydrogen phosphate and potassium nitrate with equal mole of NH_4^+ concentrations on acetoin fermentation of *B. subtilis* SF4-3 in shake flasks. The initial glucose concentration was 150 g/L. The nitrogen sources were composed of YE (5.0 g/L) and CSD (5.0 g/L). The initial pH value was adjusted to 7.00.

production (32.1 g/L) along with 21.8 g/L of 2,3-butanediol was obtained after 96 h of cultivation. When cells were cultivated in the medium containing diammonium hydrogen phosphate, the pH quickly fell to below 5.00 and the cell growth was severely suppressed (OD_{600} = 4.91 at 24 h). After 48 h, cells stopped consuming the glucose in the fermentation broth. Thus, at the end of fermentation, only 14.0 g/L of acetoin and 4.6 g/L of 2,3-butanediol were obtained. As for potassium nitrate, after the fermentation began, its pH increased continuously and rapidly rises to over 7.00 (<8.00). Although, the excessive pH had no bad effect on the cell growth, but it exerted a negative influence on the acetoin and 2,3-butanediol synthesis. According to the literature, the optima pH value for ALS and ALDC was between 6.0 and 7.0 [29]. Thereby, it was inferred that the high pH caused by the potassium nitrate containing medium

Table 2
Effect of urea concentration on acetoin and 2,3-butanediol production by *B. subtilis* SF4-3. The initial glucose concentration was 150 g/L. The complex nitrogen sources were composed of YE (10.0 g/L) and CSD (5.0 g/L). The initial pH value was adjusted to 7.00.

Urea added (g/L)	48 h			72 h		
	AC (g/L)	BD (g/L)	AC/BD	AC (g/L)	BD (g/L)	AC/BD
Control	14.4 ± 0.5	10.2 ± 0.7	1.41	20.8 ± 0.2	14.5 ± 1.0	1.43
0.30	18.4 ± 0.6	11.9 ± 0.8	1.55	23.8 ± 0.4	14.7 ± 0.8	1.62
0.90	20.1 ± 0.2	28.0 ± 0.9	0.72	35.1 ± 0.6	23.0 ± 0.7	1.53
1.50	21.9 ± 0.3	33.6 ± 1.0	0.65	36.2 ± 0.5	28.3 ± 0.9	1.28
2.00	18.9 ± 0.6	37.6 ± 0.7	0.50	39.9 ± 0.2	14.7 ± 0.7	2.71
2.50	18.6 ± 0.2	39.9 ± 0.6	0.47	35.6 ± 0.5	18.4 ± 0.6	1.93
3.00	16.4 ± 0.1	42.3 ± 0.8	0.39	34.8 ± 0.2	18.7 ± 0.8	1.86

may weaken enzyme activities of ALS or ALDC. As a result, the final concentrations of acetoin and 2,3-butanediol achieved in 96 h of cultivation was only 24.6 g/L and 18.6 g/L.

From the above results, it was clearly shown that urea was an appropriate inorganic nitrogen source substitution of high-cost organic nitrogen sources for acetoin production of *B. subtilis* SF4-3. First, urea could be used as an effective alternative of organic sources for cell growth and acetoin synthesis. Second, it also could serve as a buffer agent for maintaining the acidic environment for acetoin production of *B. subtilis* SF4-3. In addition, it was found that there was not apparent transformation of acetoin and 2,3-butanediol in the urea containing medium during the whole fermentation process, and it was suspected that this was caused by the insufficient nitrogen source supplement. Therefore, effect of urea content on acetoin production was further investigated using the enhanced complex nitrogen source medium (YE 10 g/L and CSD 5 g/L) in shake flask, and the results were shown in Table 2. Different concentrations of urea were added into the medium at the beginning of cultivation, respectively. After 48 h of cultivation, the maximum concentrations of 2,3-butanediol was achieved (42.3 g/L) with the 3.0 g/L of urea containing medium. After 72 h of fermentation, the highest reversible transformation of 2,3-butanediol happened to the medium supplement with 2.0 g/L of urea and acetoin concentration was increased to its peak value (39.92 g/L). Further increase of urea concentrations could not improve the transformation and acetoin concentration. Therefore, the most appropriate addition of nitrogen source was obtained (YE 10 g/L, CSD 5 g/L, urea 2.0 g/L).

3.4. Effect of glucose concentrations on the fermentation of *B. subtilis* SF4-3

Acetoin is an intermediate compound of microbial overflow metabolism when the bacteria are cultivated in an environment containing an excessive glucose or other fermentable carbon sources [1]. Therefore, carbon sources could be a potential factor involving in the transformation of acetoin and 2,3-butanediol. Thus, the effect of different concentrations of initial glucose addition on the transformation of acetoin and 2,3-butanediol of *B. subtilis* SF4-3 were investigated under the optimized nitrogen sources. As shown in Fig. 5, a suitable ratio of carbon and nitrogen sources was of great importance for acetoin production of *B. subtilis* SF4-3. When 50 g/L of glucose was added, it was rapidly exhausted at 24 h and further incubation led to simultaneous decline of acetoin and 2,3-butanediol, which indicated that they were reused as carbon source for maintaining cell growth. With further increase of glucose concentration (<180 g/L), reversible transformation of 2,3-butanediol to acetoin occurred and acetoin concentration increased in proportion to glucose concentrations. The maximum acetoin production (39.9 g/L) was obtained at 72 h in the medium containing 150 g/L of glucose. But, when the added glucose contents exceeded 180 g/L, the concentrations of acetoin and 2,3-butanediol obtained were slightly declining. Also, the reversible of transformation 2,3-butanediol to acetoin did not emerge throughout the whole process. On the one hand, excessive glucose addition may cause high osmotic pressure which readily led to cell dehydration and inhibit its growth. The inference could be supported by low cell mass in the fermentation broth. On the other hand, it was presumably that the high glucose supplement could afford the NADH demand for cell metabolism, thus reversible transformation of 2,3-butanediol to acetoin for NADH regeneration would not be a necessary step [2]. Ultimately, the appropriate C:N ratio was of great importance for reversible transformation of 2,3-butanediol in *B. subtilis* SF4-3 for enhancing acetoin production.

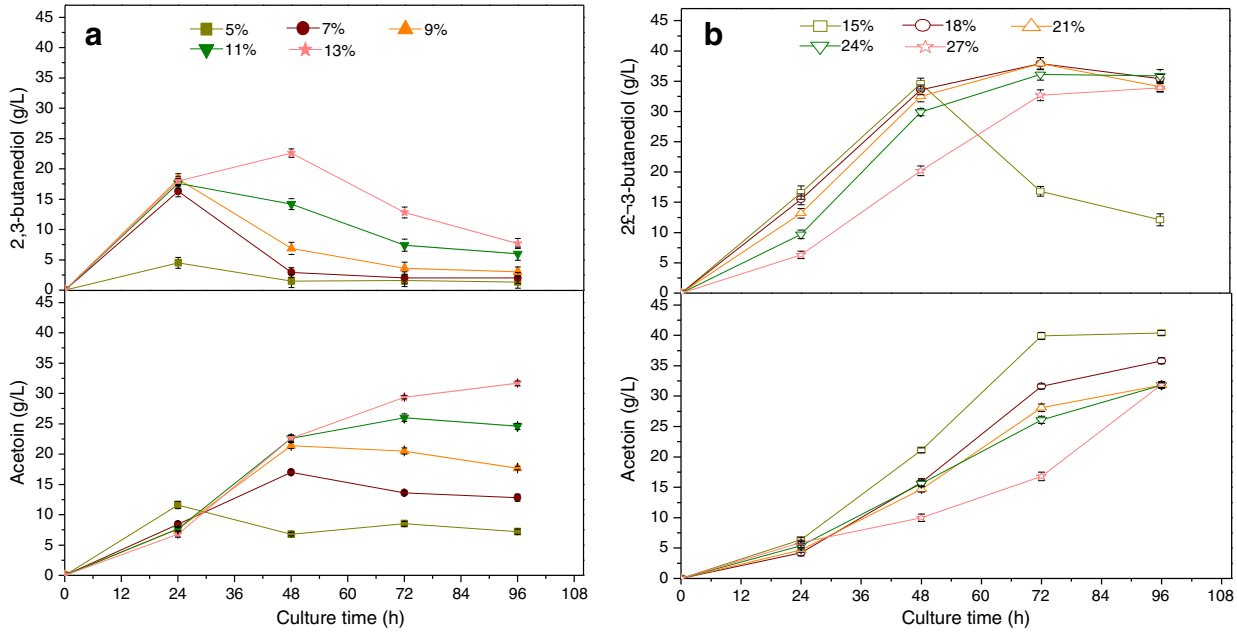

Fig. 5. Effect of initial glucose concentration on acetoin and 2,3-butanediol production by *B. subtilis* SF4-3 in shake flasks. The nitrogen sources were composed of YE (10.0 g/L), CSD (5.0 g/L) and urea (2 g/L). The initial pH value was adjusted to 7.00.

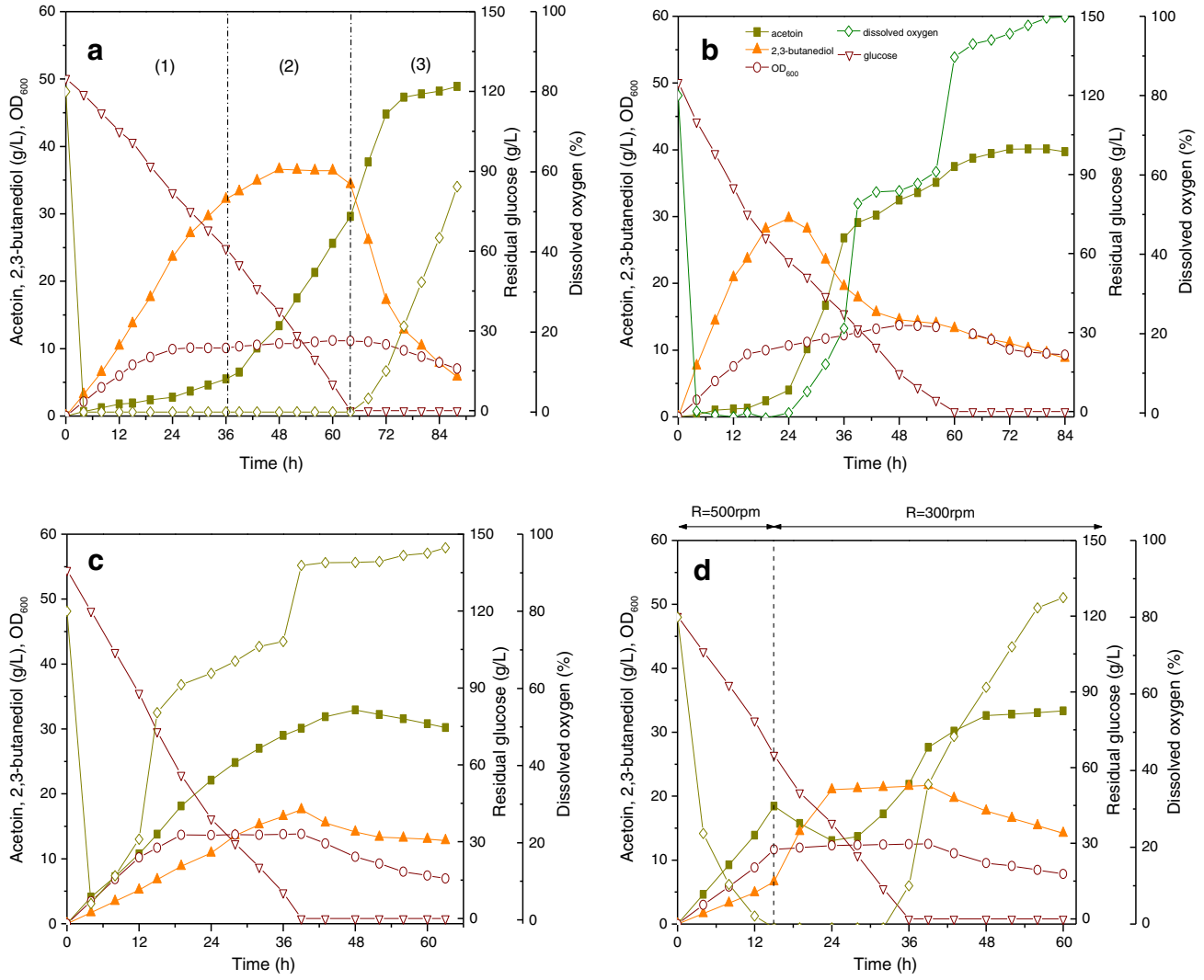

Fig. 6. Time course of acetoin fermentation by *Bn subtilis* SF4-3 at different agitation speeds in a 5-L fermenter: a (300 r/min); b (400 r/min); c (500 r/min); d (500 r/min–300 r/min). The initial glucose concentration was 150 g/L. The nitrogen sources were composed of YE (10 g/L), CSD (5 g/L) and urea (2 g/L). The initial pH value was not adjusted.

3.5. Effect of dissolved oxygen on acetoin fermentation of B. subtilis SF4-3

Moes et al. [30] found that the dissolved oxygen levels in the culture of *B. subtilis* had an important impact on the distribution of metabolites, when the dissolved oxygen level in the fermentation broth was greater than 100 parts per billion (ppb), acetoin was mainly produced; when the dissolved oxygen levels was below 100 ppb, 2,3-butanediol production was dominant [30]. Therefore, dissolved oxygen was evaluated to be associated with the transformation of acetoin and 2,3-butanediol in *B. subtilis* SF4-3 [31,32]. As well as, the effect of nitrogen source on acetoin production of *B. subtilis* SF4-3 was also involved with dissolved oxygen [31]. Therefore, during the batch fermentation in a 5-L fermenter, the effect of different dissolved oxygen supply conditions on the transformation of acetoin and 2,3-butanediol in *B. subtilis* SF4-3 were investigated by using different agitation speed control strategies of 300 r/min, 400 r/min and 500 r/min. The results indicated that the agitation speed indeed played a crucial role in the transformation of acetoin and 2,3-butanediol in *B. subtilis* SF4-3 (Fig. 6a–c). The maximum acetoin concentration of 48.9 g/L and 5.8 g/L of 2,3-butanediol was obtained at the agitation speed of 300 r/min (Fig. 6a). As agitation speed increased, concentration of acetoin decreased and 2,3-butanediol concentration increased accordingly (at 400 r/min: acetoin 39.7 g/L and 2,3-butanediol 8.8 g/L, Fig. 6b; at 500 r/min: acetoin 30.2 g/L and 2,3-butanediol 12.8 g/L, Fig. 6c). The effect of four different agitation speed control strategies on acetoin fermentation of *B. subtilis* SF4-3 was compared and summarized in Table 3. The result showed that a reasonable agitation speed was of significance to acetoin accumulation in *B. subtilis* SF4-3. In order to further verify the effect of agitation speed on the acetoin and 2,3-butanediol production, the two-stage agitation speed control strategy (500–300 r/min) was applied in

Table 3
Comparison of parameters in batch fermentation of *B. subtilis* SF4-3 at different agitation speeds.

Parameters	Agitation speed (r/min)				
	200	300	400	500	500-300[a]
Acetoin (g/L)	No results	48.9	39.7	30.2	33.4
2,3-butanediol (g/L)		5.8	8.8	12.8	14.2
Glucose consumption rate (g/L/h)[b]		1.95	2.08	3.21	3.47
Acetoin productivity (g/L/h)		0.56	0.47	0.48	0.57
Fermentation time (h)[c]		88	84	63	60
Conversion rate (%)		39.12	31.76	24.16	26.72

[a] Agitation speed was maintained at 500 r/min in the first 16 h, and then it was changed to 300 r/min till the end.
[b] The time was defined as the time when the glucose was exhausted.
[c] The fermentation time was defined the time when the glucose was exhausted plus extra 24 h for transformation of 2,3-butanediol.

the fermentation process. During the first agitation speed of 500 r/min (16 h), production of acetoin was superior to 2,3-butanediol. However, when the agitation speed was switched to 300 r/min, acetoin production started to decline for a while, and then it started to increase gradually until the end of fermentation. Meanwhile, 2,3-butanediol production appeared large enhancement and tended to be a steady state until its sudden decline. Finally, no desired acetoin production was obtained in comparison with that at 300 r/min (acetoin 33.4 g/L, 2,3-butanediol 14.2 g/L, Fig. 6d).

When the agitation speed was controlled at 300 r/min, the entire fermentation process could be divided into three stages (Fig. 6a). In the first stage (0–36 h), most of glucose was transformed to 2,3-butanediol and acetoin concentrations increased slowly. At the second stage (36–64 h), the production of 2,3-butanediol enter into a steady state while acetoin concentration started to increase dramatically. In this phase, the NADH generated from glycolytic pathway may mostly flow into the electron transfer chain, so the produced acetoin would not be transformed to 2,3-butanediol for NAD regeneration [33]. Meanwhile (0–64 h), dissolved oxygen firstly rapidly drop to 0% at 4 h and kept below 0% for the rest of the time. Complete exhausting of glucose in the medium accompanied by the sudden ascent of dissolved oxygen indicated the advent of the third stage (64–87 h), 2,3-butanediol started to conversely transformed to acetoin for NADH recycle: 2,3-butanediol concentration declined sharply, and acetoin concentration persistently increased to be a steady state. Lower agitation speed (200 r/min) would limit cell growth, and even without consuming the glucose in the medium (the results not shown). When the agitation speeds were increased to 400 r/min or 500 r/min, adequate dissolved oxygen supply led to reduction of 2,3-butanediol accumulation and improvement of acetoin concentration. Compared with the result at 300 r/min, reversible transformation of 2,3-butanediol to acetoin occurred ahead of time (400 r/min) and even disappeared (500 r/min). The results may be explained by the fact that the high agitation speed readily caused high dissolved oxygen, so the enhanced aerobic respiration via the electron transfer chain would consume the majority of NADH, which would decrease the metabolic flux to acetoin and 2,3-butanediol pathway. The two-stage agitation speed experiment further indicated that it was crucial to keep a reasonable agitation speed for acetoin production in *B. subtilis* SF4-3.

4. Conclusions

The transformation of acetoin and 2,3-butanediol was discovered in an acetoin-producing strain, *B. subtilis* SF4-3. In order to achieve high acetoin production, the reversible transformation of 2,3-butanediol for acetoin production was investigated in this paper. Medium components (nitrogen sources, carbon sources) and dissolved oxygen were found to play important roles in the reversible transformation of 2,3-butanediol to acetoin in *B. subtilis* SF4-3. From the perspective of economics and production of acetoin fermentation, the optimal medium (glucose·H_2O 150 g/L, YE 10 g/L, CSD 5 g/L, urea 2 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.5 g/L) were obtained. Furthermore, the effect of dissolved oxygen on acetoin production was explored in a 5-L fermenter via different agitation speeds. A reasonable agitation speed was proved to be significant for reversible transformation of 2,3-butanediol for acetoin production, and the maximum acetoin production (48.9 g/L) with conversion rate of 39.12% (g/g) was obtained at the agitation speed of 300 r/min.

Compared with the reported acetoin production in other bacterial acetoin production system, 48.9 g/L of acetoin production in this manuscript do not seem dominant. But, the most important originality of this manuscript is that it provides a method for acetoin production based on the reversible transformation of acetoin and 2,3-butanediol. Also, 39.12% of conversion rate indicates *B. subtilis* SF4-3 is a competitive strain whose acetoin production could be further enhanced by means of elaborate agitation speed and fed-batch feeding strategy. In addition, the simple operation process contributes to its feasibility in large scale acetoin production. Currently, 100-L scale of acetoin pilot fermentation has been finished in our laboratory and its production reaches a relatively steady level of 50–60 g/L at a time of 90–96 h. Furthermore, 1000-L scale of acetoin fermentation would be achieved in the near future. Now, there still is not acetoin produced by microbial fermentation on the market. Its industrialization will bring enormous economical benefits and social effects.

Conflict of interests

None.

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