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Acetoin production enhanced by manipulating carbon flux in a newly isolated *Bacillus amyloliquefaciens*

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HIGHLIGHTS

- An acetoin high-producing *Bacillus amyloliquefaciens* FMME044 was newly isolated.
- The mechanism of exchange of 2,3-butanediol and acetoin is explained.
- A novel strategy of manipulating carbon flux to acetoin is proposed.
- 51.2 g/L acetoin with yield of 0.43 g/g and productivity of 1.42 g/L/h was achieved.

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ABSTRACT

A new strain, FMME044, exhibited a remarkable ability to synthesize acetoin and was identified as *Bacillus amyloliquefaciens*. The following characteristics of enzyme activity were found: 2,3-butanediol was reverse transformed to acetoin upon depletion of glucose; lower agitation speeds favored 2,3-butanediol accumulation; and higher agitation speeds favored reverse transformation of 2,3-butanediol to acetoin. In order to enhance acetoin production by manipulating the carbon flux distribution, a two-stage agitation speed control strategy was proposed: during the first 24 h, the agitation speed was set to 350 rpm to achieve a high 2,3-butanediol concentration and then the speed was increased to 500 rpm to reverse transform 2,3-butanediol to acetoin. Following this strategy, a high titer (51.2 g L^{-1}), yield (0.43 g g^{-1}), and productivity (1.42 g L^{-1} h^{-1}) of acetoin were achieved. The results demonstrated that *B. amyloliquefaciens* FMME044 is a potential industrial strain for acetoin production.

1. Introduction

Acetoin is a flavoring compound that is naturally found in fresh apples, milk, wines etc., and widely used in food production and as a chemical raw material and precursor in the synthesis of liquid fuels by microorganisms (Singh and Krishnan, 1959). At present, there are three methods of industrial production of acetoin, including microbial fermentation, enzymatic conversion, and chemical synthesis (Toda et al., 1989). Compared with the chemical method and enzymatic conversion, microbial fermentation is a cost-effective approach with many advantages, including inexpensive raw materials, less environmental pollution, high purity of products, and mild reaction conditions.

It is believed that a high-yielding strain would play a vital role in the commercial fermentation of acetoin. Many microorganisms have the ability to produce acetoin, including *Saccharomyces cerevisiae* (Romano et al., 1993), *Saccharomyces carlsbergensis* (Tomita et al., 1969), *Hanseniaspora guilliermondii* (Teixeira et al., 2002), *Lactococcus lactis* (Bassit et al., 1993), *Lactobacillus casei* (Branen and Keenan, 1971), *Leuconostoc citrovorum* (Collins and Speckman, 1974), and *Zygosaccharomyces bailii* (Romano and Suzzi, 1993). Three other strains, *Serratia marcescens* H13 (75.2 g L^{-1}) (Sun et al., 2012a), *Bacillus licheniformis* MEL 09 (41.2 g L^{-1}), (Liu et al., 2011a), and *Bacillus pumilus* DSM 16187 (63.0 g L^{-1}, EP 2005081882), exhibited high acetoin-producing ability from glucose. In bacteria, much research has focused on the following areas: (1) elucidation of the metabolic control mechanisms (Nicholson, 2008); (2) manipulation of the carbon flux to reduce the production of byproducts (Sun et al., 2012b); (3) isolation of hyper-producing strains (Liu et al., 2011a); and (4) production of highly pure stereoisomeric 2,3-butanediol and acetoin using resting cells (Liu et al., 2011b). In this study, a strain demonstrating a high productivity of acetoin was isolated from the soil and identified as *Bacillus amyloliquefaciens* according to its 16S rDNA

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sequence and *gyrA* gene analysis as well as the results of physiological and biochemical experiments. *B. amyloliquefaciens* is a GRAS (Generally Recognized As Safe) strain (Yang et al., 2011), and its volatile metabolites have strong antifungal effects (Arrebola et al., 2010), so *B. amyloliquefaciens* has been widely used in industrial fermentation. Furthermore, the acetoin-producing capability of *B. amyloliquefaciens* could be further enhanced by optimizing the nutrient conditions and manipulation of the carbon flux during fermentation.

2. Methods

2.1. Media and culture conditions

The medium used for isolation of the acetoin-producing strain (medium A) contained 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 5 g L^{-1} NaCl. Medium B contained 5 g L^{-1} glucose, 5 g L^{-1} peptone, and 5 g L^{-1} K_2HPO_4 for the Voges–Proskauer (VP) test. The medium used for seed culture (medium C) contained: 60 g L^{-1} glucose, 10 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} beef extract, and 5 g L^{-1} NaCl (Xiao et al., 2007). The fermentation medium (medium D) contained 100 g L^{-1} glucose, 20 g L^{-1} yeast extract, 0.2 g L^{-1} $MgSO_4$, 3 g L^{-1} K_2HPO_4, 3 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl. The optimum fermentation medium (medium E) contained 120 g L^{-1} glucose, 10 g L^{-1} yeast extract, 10 g L^{-1} peptone, 0.2 g L^{-1} $MgSO_4$, 3 g L^{-1} K_2HPO_4, 3 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (peptone and yeast extract used in the experiment contain 1 g L^{-1} nitrogen, respectively).

The seed culture was cultivated in a 500-mL flask containing 50 mL medium C on a rotary shaker for 12 h. Fermentaion was carried out in a 7-L jar fermentor (BioFlo 410-7L, New Brunswick Scientific, Enfield, CT, USA) with 4 L of medium E. The inoculation size was 10% (v/v) in the fermentation medium; the pH was not controlled; the agitation speed and aeration rate were controlled at 400 rpm and 4 L min^{-1}, respectively. The flask culture was grown for 60 h and the rotation rate was controlled at 200 rpm. All experiments were carried out in triplicate. All cultivations were performed at 37 °C.

2.2. Isolation and identification of the acetoin-producing strain

Of the 54 samples that were collected from soil, distiller's grains, and agricultural residues in Jiangsu Province, China, 1-g portions were weighed out and added to bottles containing 100 mL of distilled water and throughly mixed. The solutions were diluted to 10^{-4}, 10^{-5}, and 10^{-6} and spread onto screening plates containing medium A. Incubation was conducted at 37 °C for 24 h. Colonies that appeared on medium A plates were inoculated into medium B for the VP test. Then, 8-mL aliquots of fermentation broth were taken between 12 and 36 h during culture in medium B at 37 °C and mixed with 1 mL 10% NaOH solution, which contained 5% β-naphthol and 1 mL 0.5% creatine, and heated at 60 °C for 15 min. Red color signified a positive result. The VP-positive strains were subsequently inoculated in medium D at 37 °C for 60 h to determine the acetoin production capability. Strains with high yields of acetoin were dissolved in 20% (v/v) glycerol at −80 °C for long-term storage.

The acetoin-producing strain was identified by its morphological, physiological, and biochemical characteristics and from its 16S rDNA sequence and *gyrA* gene analysis. The acetoin-producing strain was observed by light microscopy to examine the morphological characteristics of the cells and spores. Physiological and biochemical identification were performed according to *Bergey's Manual of Systematic Bacteriology*. The 16S rDNA sequence was obtained as described by Xu and Cote (2003). The results of sequence analysis were compared with sequences in the GenBank databases using the BLAST program. The gyrA gene analysis was conducted using the method described by Roberts et al. (1994).

2.3. Resting cells experiment

Cells at the mid-exponential growth phase dry cell weight (DCW) = 13–15 g L^{-1}, the time at which glucose was exhausted) were collected and centrifuged (10,000 rpm at 0 °C for 10 min). The harvested cells were washed twice with saline solution (0.9% NaCl). Resting cells were inoculated into a 500-mL flask containing 50 mL of phosphate buffer (pH 7.0) and 2,3-butanediol was used as substrate. The biotransformation was carried out at 37 °C and 200 rpm on a rotary shaker.

2.4. Assay methods

Qualitative analysis of acetoin was determined by GC–MS. The concentrations of acetoin and 2,3-butanediol were determined using an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and a refractive index detector (RID). The injection volume was 20 μL and 5 mmol L^{-1} H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. The column temperature was 60 °C. Glucose was assayed enzymatically using a commercial test kit supplied by Merck (Merckotest 14365; Merck, Whitehouse station, NJ, USA). Cell concentration was expressed as DCW. The harvested cells were washed twice with distilled water, resuspended, and centrifugation again, and then the sediment was dried at 80 °C to a constant weight.

3. Results and discussion

3.1. Isolation and identification of the acetoin-producing strain

In this study, 54 collected samples were washed with distilled water and spread onto medium A. Colonies that appeared on medium A plates were inoculated into medium B for the Voges–Proskauer (VP) test. 27 colonies that exhibited a positive VP test in medium B were selected and inoculated into medium C to test for acetoin-producing capability. It was found that four strains could secrete more than 20 g L^{-1} acetoin, and the colony that secreted the highest quantity, 23.8 ± 0.5 g L^{-1}, was named FMME044. Furthermore, the main metabolite of FMME044 was identified as acetoin by GC–MS, using acetoin as the standard compound.

On LB plates, the colonies of strain FMME044 were cream in color and opaque and exhibited a wrinkled surface. FMME044 cells were rod-shaped, gram-positive, and motile, and bore a central/subterminal ellipsoidal spore. Physiological and biochemical experiments demonstrated that FMME044 was aerobic, catalase-positive, nitrate reduction-positive, indole-positive, capable of starch and gelatin hydrolysis, and methyl red-negative. All these characteristics supported the identification of this strain as a member of the genus *Bacillus*. Furthermore, the 16S rDNA of FMME044 (Genbank accession number: JN882263.1) exhibited 99.0% identity to that of *Bacillus amyloliquefaciens* NBRC 15535. In order to further distinguish FMME044 from the *B. subtilis* group, the *gyrA* gene of FMME044 was isolated and showed 99%, 84%, 79%, and 77% homology with *B. amyloliquefaciens* DSM 7, *Bacillus subtilis subsp. spizizenii* TU-B-10, *Bacillus licheniformis* ATCC 14580, and *Bacillus cereus* BDRD-Cer4, respectively. These results demonstrated that strain FMME044 belonged to *B. amyloliquefaciens*, and it was consequently named *B. amyloliquefaciens* FMME044. The *gyrA* is the gyrase A (GyrA) gene; GyrA accumulates amino acid substitutions in a random molecular clock fashion without any significant

evolutionary pressure (Chun and Bae, 2000). Therefore, the *gyrA* sequence is usually used to accurately classify *B. subtilis* and related taxa (Yssel et al., 2011).

3.2. Effect of nutrition on acetoin production by FMME044

The effect of the carbon source (glucose, fructose, sucrose, lactose, maltose, xylose, and sorbose, all at 100 g L^{-1}) on cell growth and acetoin production was investigated. It was found that strain FMME044 can utilize all of the above sugars as carbon and energy sources for cell growth. However, only glucose, sucrose, and fructose can be converted to acetoin, and the corresponding acetoin titers were 35.9 g L^{-1}, 32.5 g L^{-1}, and 20.2 g L^{-1}, respectively. Among these, the highest DCW (9.7 g L^{-1}) and acetoin concentration (35.9 g L^{-1}) were achieved with 100 g L^{-1} glucose as the carbon source. Based on the above results, the effects of the initial glucose concentration on cell growth and acetoin production were further explored. The cell growth increased as the initial glucose concentration increased from 80 g L^{-1} to 140 g L^{-1}, but no further increases were observed when more than 160 g L^{-1} glucose was added to the fermentation medium. The acetoin titer increased from 30.5 to 40.2 g L^{-1} when the initial glucose concentration increased from 80 to 120 g L^{-1}. However, compared to the case when 120 g L^{-1} glucose was added, lower acetoin production was detected when more than 140 g L^{-1} glucose was present in the medium. The reasons for the lower acetoin production may be related to insufficient nitrogen in the medium or a decrease in cell growth. Furthermore, the glucose supply was completely exhausted when less than 140 g L^{-1} glucose was added to the fermentation medium initially. Therefore, 120 g L^{-1} glucose was used for the following experiments.

The effects of the nitrogen source (the content of nitrogen is 2 g L^{-1}, including yeast extract, peptone, tryptone, corn steep liquor, urea, ammonium chloride, ammonium sulfate, ammonium acetate, sodium nitrate and ammonium nitrate) on cell growth and acetoin production were investigated. When strain FMME044 was grown on an inorganic nitrogen source, the cell growth and acetoin production were generally lower than the corresponding values when an organic nitrogen source was used. Among of the organic nitrogen sources, more than 38.0 g L^{-1} acetoin was achieved when yeast extract or peptone as sole nitrogen source, and the highest cell growth (13.8 g L^{-1}) and acetoin production (40.2 g L^{-1}) were obtained with yeast extract, but the maximum yield of acetoin per cell growth (3.3 g L^{-1}) was observed when peptone was the nitrogen source. These results demonstrated that yeast extract and peptone were not only suitable nitrogen sources, but also could offer some growth factors to stimulate cell growth. In order to further enhance the efficiency of acetoin production, a mixed nitrogen source containing 10 g L^{-1} peptone and 10 g L^{-1} yeast extract was used, and the highest cell growth (14.6 g L^{-1}) and acetoin production (46.0 g L^{-1}) were achieved, higher by 13.0%, 14.4% and 33.3%, 19.8% than the corresponding values when yeast extract and peptone were used as the sole nitrogen source, respectively.

The studies indicated that 2 g L^{-1} nitrogen was needed for cell growth and to fully metabolize 120 g L^{-1} glucose within 48 h (data not shown). These results meant that a suitable ratio of carbon to nitrogen (C:N) in the fermentation medium played a key role in acetoin production. The effect of the C:N ratio on acetoin production was investigated. It seems that both cell growth and acetoin production are maximized at a C:N ratio of 24:1. When the C:N ratio was either increased to 28:1 or decreased to 20:1, acetoin production decreased by 11.3% and 8.3%, respectively. The above results strongly suggested that the optimum ratio of C:N was 24:1. However, when the concentrations of the carbon and nitrogen sources were increased proportionally keeping the ratio of C:N constant, the acetoin concentration decreased by 9.6% but cell growth increased by 8.2%.

3.3. Acetoin production by B. amyloliquefaciens FMME044 in a 7-L fermentor

The time-course of acetoin production by *B. amyloliquefaciens* FMME044 in a 7-L fermentor is shown in Fig. 1. A series of conclusions could be drawn from Fig. 1: (1) the lag phase of cell growth was shorter than 6 h, and the DCW reached its maximum value (15.8 g L^{-1}) at 16 h; (2) only two metabolites, acetoin and 2,3-butanediol, were detected during the first 4 h; (3) the titers of 2,3-butanediol gradually increased to the highest value (30.7 g L^{-1}) at 16 h, then began to decline to 5.9 g L^{-1} at 48 h; (4) at 48 h, the titer of acetoin reached 50.1 g L^{-1} with 120 g L^{-1} glucose (to the best of our knowledge, this titer is the highest value achieved in a 7-L fermentor by *B. amyloliquefaciens* FMME044); (5) compared with that of 2,3-butanediol, a higher production rate of acetoin was observed.

3.4. Manipulating the carbon flux from 2,3-butanediol to acetoin by using an oxygen control strategy

As illustrated in Fig. 1, an interesting phenomenon was observed in that the concentration of 2,3-butanediol decreased with the depletion of glucose in the fermentation broth, but the acetoin concentration increased. This result indicated that 2,3-butanediol may be converted to acetoin when the glucose supply has been exhausted. To investigate this, *B. amyloliquefaciens* FMME044 cells were harvested at mid-exponential growth phase (DCW = 13–15 g L^{-1}) and then added to biotransformtion broths, which contained 5 g L^{-1}, 10 g L^{-1}, and 20 g L^{-1} 2,3-butanediol. After 6 h, the 2,3-butanediol was completely converted to 4.5 g L^{-1}, 9.1 g L^{-1}, and 17.2 g L^{-1} acetoin, respectively (Syu, 2001). How is *B. amyloliquefaciens* FMME044 able to convert 2,3-butanediol to an equimolar quantity of acetoin? The metabolic map of *B. amyloliquefaciens* demonstrates that glucose is metabolized to pyruvate by glycolysis with the concomitant production of NADH; then pyruvate is further converted to acetoin to maintain pH homeostasis (Tsau et al., 1992). In order to provide enough NAD^+ for the glycolytic pathway, acetoin is converted to an equimolar quantity of 2,3-butanediol in the presence of 2, 3-butanediol dehydrogenase and NADH is oxidized to NAD^+. With the depletion of glucose, 2,3-butanediol is reversibly converted to acetoin to regenerate NADH

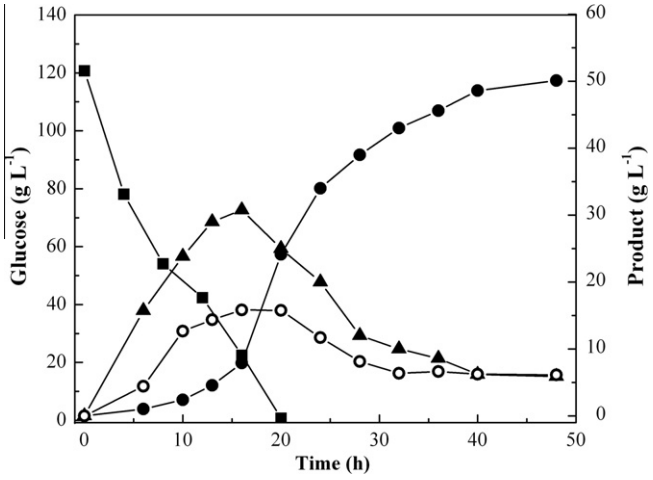

Fig. 1. Time course of acetoin fermentation by *B. amyloliquefaciens* FMME044 in a 7-L fermentor. (■) Glucose; (●) acetoin; (▲) 2,3-butanediol; (○) DCW.

to maintain a constant oxidation–reduction state after glycolysis has ceased and to provide a carbon source for cell growth. Sun et al. (2012a) expressed the *nox* gene encoding a water-forming NADH oxidase to increase the intracellular NAD^+ concentration, and found that production of 2,3-butanediol was depressed and accumulation of acetoin was enhanced. The essence of the interchange of 2,3-butanediol and acetoin is the oxidation and regeneration of NADH. In view of this, the metabolic characteristics of 2,3-butanediol and acetoin in our system at different oxygen levels (agitation at 300 rpm to 500 rpm) were investigated and the results are depicted in Fig. 2. As illustrated in Fig. 2A, the titer of 2,3-butanediol increased with decreasing agitation speed, and the highest titer of 2,3-butanediol (58.1 g L^{-1}) was achieved at 300 rpm, higher by 9% (350 rpm), 88.9% (400 rpm), and 193% (500 rpm) than the corresponding values at higher agitation speeds. With the depletion of glucose, the 2,3-butanediol concentration began to decline (Fig. 2B). Meanwhile, the concentration of acetoin increased with increasing agitation speed (less than 400 rpm), and the highest acetoin concentration (50.1 g L^{-1}) was acquired at 400 rpm (Fig. 2C). Although the acetoin concentration at 400 rpm was higher by 32.9% than that of the 500 rpm case, the concentration of the residual 2,3-butanediol at 500 rpm (3.2 g L^{-1}) was 45.8% less than that at 400 rpm (5.9 g L^{-1}). This suggested that the higher agitation speed (500 rpm) promoted the reverse transformation of 2,3-butanediol to acetoin. In addition, as Fig. 2D shows, enhancement of the agitation speed caused dissolved oxygen (DO) to decrease faster. Based on the above results, a two-stage agitation speed control strategy was proposed (Fig. 3): the agitation speed was controlled at 350 rpm during the first 24 h and then switched to 500 rpm. It was found that, when the agitation speed was switched to 500 rpm (24 h), DO rose to a value that was 212.5% higher than at 350 rpm (24 h). By following the optimum control strategy, the concentration of 2,3-butanediol gradually increased to 53.7 g L^{-1} at 24 h and 51.2 g L^{-1} acetoin accumulated in the fermentation broth by 48 h. The production of acetoin was higher by 46% than the yield for *B. amyloliquefaciens* B10-127 (Yang et al., 2012). Similarly, the yield and productivity were 0.43 g g^{-1} and 1.42 g L^{-1} h^{-1}, higher by 13.2% and 49.5%, respectively, than when a single agitation speed of 350 rpm was adopted. A high concentration of 2,3-butanediol accumulated at low DO levels due to the fact that: (1) the alsSD operon (encoding acetolactate synthase and decarboxylase) was up-regulated (Yu et al., 2011); (2) a lower ratio of NAD^+/NADH was conducive to activating acetoin reductase, so more carbon flux was channeled into 2,3-butanediol. As the DO concentration rose, more NADH was oxidized to NAD^+ through oxidative phosphorylation; in order to maintain the intracellular NADH concentration at a constant level, the specific activity of 2,3-butanediol dehydrogenase was enhanced and, as a result, more 2,3-butanediol was reverse transformed to acetoin.

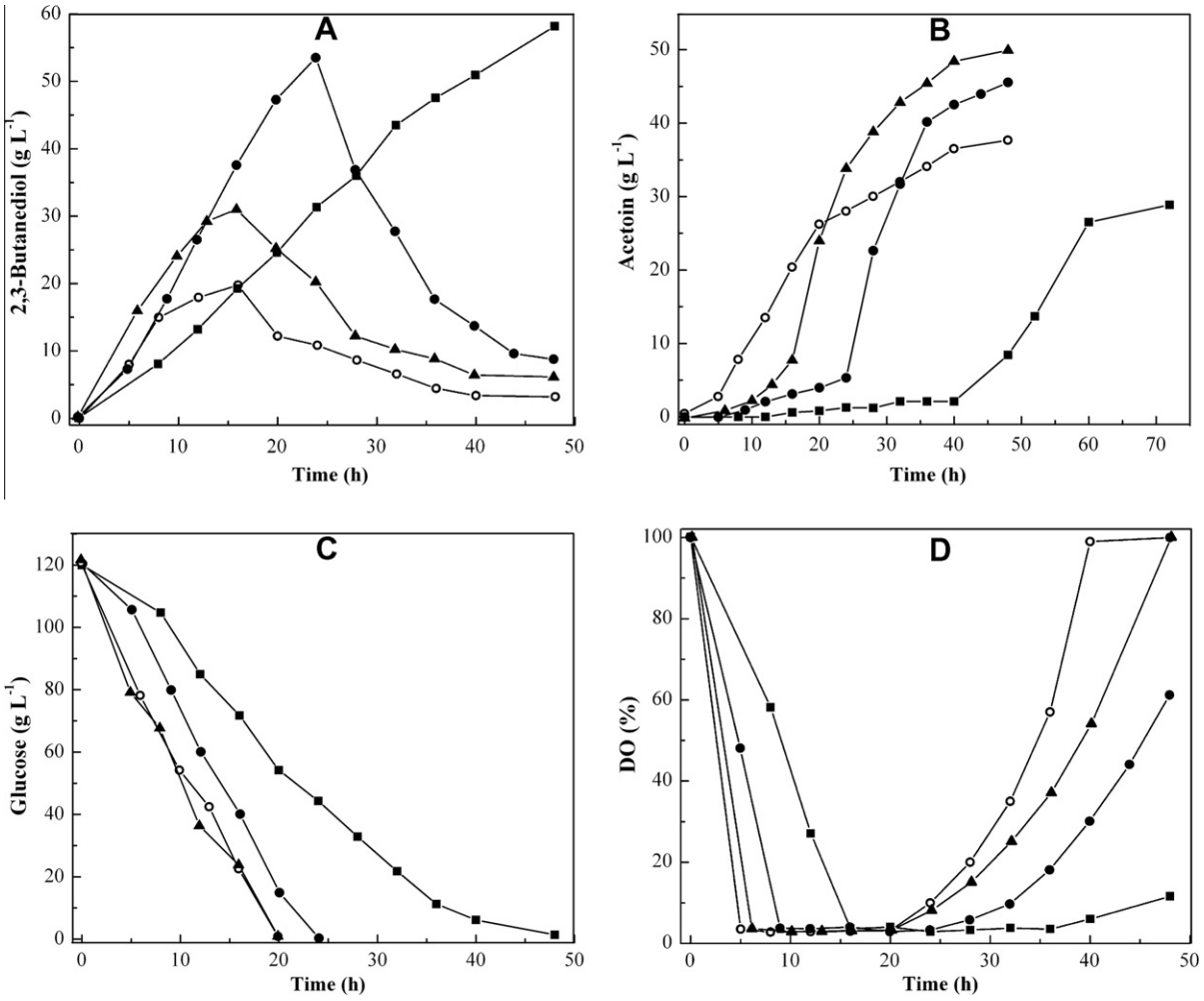

Fig. 2. Effect of agitation speed on acetoin fermentation. (■) 300 rpm; (●) 350 rpm; (▲) 400 rpm; (○) 500 rpm.

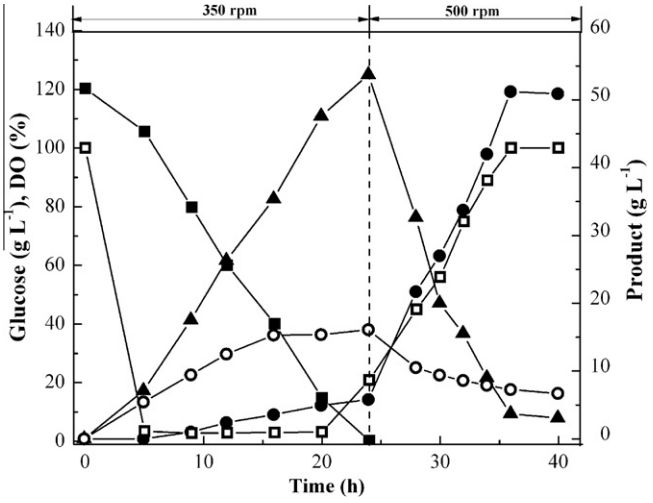

Fig. 3. Time course of acetoin fermentation by *B. amyloliquefaciens* FMME044 when the two-stage agitation speed control strategy was used: 350 rpm for the first 24 h then switched to 500 rpm. (■) Glucose; (●) acetoin; (▲) 2,3-butanediol; (○) DCW; (□) DO.

4. Conclusions

In this study, enhancement of acetoin production was achieved by the newly isolated *B. amyloliquefaciens* FMME044. An acetoin concentration of 51.2 g L^{-1} with a yield of 0.43 g g^{-1} and productivity of 1.42 g L^{-1} h^{-1} were achieved by manipulating the carbon flux distribution and utilizing a two-stage agitation speed control strategy. This strategy consisted of an agitation speed of 350 rpm during the first 24 h to achieve a high 2,3-butanediol concentration, followed by a switch to 500 rpm to reverse transform 2,3-butanediol to accumulate acetoin. This study has provided a novel strategy for industrial acetoin fermentation.

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