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RESEARCH PAPER

Enhanced Acetoin Production by *Serratia marcescens* H32 Using Statistical Optimization and a Two-stage Agitation Speed Control Strategy

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Abstract Enhanced acetoin production was carried out by *Serratia marcescens* H32. First, medium compositions were optimized statistically for shake flask fermentations to produce acetoin. Sucrose and corn steep liquor powder (CSLP) were identified as the most significant factors by Plackett–Burman design. The path of steepest ascent and response surface methodology were then employed to determine the optimal concentrations of the two factors. Acetoin yield was up to 41.5 g/L in flask fermentations using the optimized medium. Furthermore, the optimal medium was used to conduct fermentation experiments in a 3.7-L bioreactor. The influences of different agitation speeds on acetoin production were investigated. Based on a process analysis, a two-stage agitation speed control strategy was proposed, in which the agitation speed was controlled at 700 rpm during the first 8 h and then switched to 600 rpm. A relatively high acetoin concentration (44.9 g/L) and high acetoin productivity (1.73 g/L/h) were achieved by applying this strategy. Fed-batch fermentation based on the two-stage agitation speed control strategy was performed, and a maximum acetoin concentration of 60.5 g/L with productivity of 1.44 g/L/h was achieved.

Keywords: acetoin, *Serratia marcescens*, medium optimization, two-stage agitation speed control strategy, fed-batch fermentation

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1. Introduction

Acetoin (3-hydroxy-2-butanone) is a high-value product that naturally occurs in wine, honey, cocoa, butter, coffee, and strawberry [1]. Acetoin is widely used not only in dairy products, but also in cosmetics and pharmacy and chemical synthesis. It has been classified as one of 30 platform chemicals that were given priority for development and utilization by the US Department of Energy [2]. Several chemical synthetic methods are available for acetoin preparation [3], but fermentative production of acetoin by microorganisms is the most environmentally friendly and cost-effective method.

Acetoin can be synthesized from carbohydrates via the mixed acid fermentation pathway by many bacterial species such as *Klebsiella pneumoniae* [4], *Klebsiella oxytoca* [5], *Bacillus amyloliquefaciens* [6], *Bacillus polymyxa* [7], *Bacillus pumilus* [1], *Enterobacter cloacae* [8], *Hanseniaspora guilliermondii* [9], *Lactococcus lactis* [10], *Lactobacillus casei* [11], *Leuconostoc mesenteroides* [12], and *Lactobacillus rhamnosus* [13]. However, acetoin is only a minor byproduct of 2,3-butanediol fermentation with most of the above strains. Thus, more efforts at medium optimization, as well as strain screening and development, have been made to improve acetoin production. *Bacillus subtilis* CICC 10025 and *Bacillus licheniformis* MEL09 show increased acetoin production ability using optimized

medium [3,14], demonstrating the importance of medium optimization. Additionally, three efficient acetoin-producing strains (*Bacillus subtilis* JNA-310 [15], *B. subtilis* TH-49 [16], and *Bacillus pumilus* DSM 16187 [17]) were obtained using natural isolation or ultraviolet (UV)/chemical mutation methods, and can also be used to produce relatively high acetoin concentrations.

Besides the above strains, *Serratia marcescens* also exhibits good potential for acetoin production when employed to produce 2,3-butanediol [30,31]. *S. marcescens* is a pigmented Gram-negative enteric organism that has been widely applied to produce prodigiosin [18,19], protease [20], pyruvate [21], L-asparaginase [22], serratiopeptidase [23,24], lipase [25,26], catalase [27,28], and molybdenum blue [29]. As *S. marcescens* is a widely used strain, it is of value to study its acetoin production characteristics.

In a previous study, oxygen supply was a critical factor for acetoin and 2,3-butanediol production. Moes *et al*. reported that dissolved oxygen (DO) level has a profound effect on product distribution during fermentation [32], with acetoin being excreted under high DO levels and 2,3-butanediol under low DO levels. Ji *et al*. studied the acetoin and 2,3-butanediol production procedure at different oxygen supply conditions by changing agitation speed [33], and similar trends were found. Thus, oxygen supply conditions should also be explored besides strain selection and medium optimization to optimize acetoin production.

In this study, a bacterium designated as *Serratia marcescens* H32, which was mutagenized and domesticated from *S. marcescens* A3 for prodigiosin production [34], was studied for acetoin production. A suitable fermentation medium component was developed using statistical methods. Then a two-stage agitation speed control strategy was proposed to efficiently produce acetoin. Finally, fed-batch fermentation was conducted, and acetoin concentration was further improved.

2. Materials and Methods

2.1. Chemicals

Corn steep liquor powder (CSLP) was purchased from Shanghai Xiwang Starch Sugar Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and were commercially available.

2.2. Microorganism and culture conditions

The strain of *S. marcescens* H32 used in this study was a high acetoin production mutant from *S. marcescens* A3 mutagenized by physical (UV) and chemical (LiCl) mutation and domesticated with an acetoin concentration gradient of 20, 30, and 40 g/L. It was maintained at 4°C on agar slants containing 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, and 20 g/L agar at pH 7.0.

The seed medium was composed of 10 g/L glucose, 2 g/L peptone, 1 g/L yeast extract, 6 g/L $(NH_4)_2SO_4$, 10 g/L K_2HPO_4, 0.5 g/L NaCl, and 0.5 g/L $MgSO_4{\cdot}7H_2O$ at pH 7.0. Seed cultivation was conducted in 250-mL shake flasks for 12 h at 200 rpm and 28°C. The prepared seed culture was inoculated (5%, v/v) into fermentation media containing the appropriate specified additions and incubated for 30 h at 200 rpm and 28°C.

2.3. Analytical methods

Sample sucrose concentrations were measured using a glucose reagent kit (Jiemen Bio-Tech Co. Shanghai, China) after centrifugation and sucrose hydrolysis.

Biomass concentration was determined by optical density (OD) at 600 nm using a spectrophotometer (UV-2008h, Unic, Mill Creek, WA, USA) and correlated with dry cell weight (DCW).

The products (acetoin and 2,3-butanediol) in the broth were extracted with ethyl acetate after adding n-butanol as the internal standard, and then quantified using a gas chromatography (GC) system (Agilent GC6890N, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies). Operation conditions were as follows: nitrogen was used as the carrier gas; injection port temperature and detector temperature were 215 and 245°C, respectively; and column oven temperature was maintained at 50°C for 1.5 min and then raised to 180°C at a rate of 25°C/min. Product concentrations were determined with calibration curves.

2.4. Statistical experimental design

A three-step experimental design based on statistical methods was used to optimize the medium for acetoin production. The Plackett–Burman (PB) design [35] was used to select factors that significantly influenced acetoin production and was based on a first-order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. Table 1 illustrates the levels of each variable and the design details. Variables with significant effects on acetoin production were used for further optimization.

The path of steepest ascent was generated by the first-order empirical equation obtained by the PB design to move towards the optimum response. The center point of the PB design was taken as the origin for the steepest

Table 1. The Plackett-Burman design matrix with acetoin production as response*

Run	Variable levels							Acetoin (g/L)
	X_1(g/L)	X_2(g/L)	X_3(g/L)	X_4(g/L)	X_5(g/L)	X_6(g/L)	X_7(g/L)	
1	+1(150)	-1(8)	+1(8)	-1(0.3)	-1(0.2)	-1(0)	+1(0.02)	35.9
2	+1(150)	+1(12)	-1(4)	+1(0.9)	-1(0.2)	-1(0)	-1(0)	32.5
3	-1(130)	+1(12)	+1(8)	-1(0.3)	+1(0.6)	-1(0)	-1(0)	28.4
4	+1(150)	-1(8)	+1(8)	+1(0.9)	-1(0.2)	+1(0.15)	-1(0)	37.6
5	+1(150)	+1(12)	-1(4)	+1(0.9)	+1(0.6)	-1(0)	+1(0.02)	32.0
6	+1(150)	+1(12)	+1(8)	-1(0.3)	+1(0.6)	+1(0.15)	-1(0)	36.4
7	-1(130)	+1(12)	+1(8)	+1(0.9)	-1(0.2)	+1(0.15)	+1(0.02)	30.6
8	-1(130)	-1(8)	+1(8)	+1(0.9)	+1(0.6)	-1(0)	+1(0.02)	29.9
9	-1(130)	-1(8)	-1(4)	+1(0.9)	+1(0.6)	+1(0.15)	-1(0)	24.5
10	+1(150)	-1(8)	-1(4)	-1(0.3)	+1(0.6)	+1(0.15)	+1(0.02)	27.5
11	-1(130)	+1(12)	-1(4)	-1(0.3)	-1(0.2)	+1(0.15)	+1(0.02)	27.4
12	-1(130)	-1(8)	-1(4)	-1(0.3)	-1(0.2)	-1(0)	-1(0)	23.0

*Factors represented constituents: X_1 (Sucrose), X_2 (Ammonium Citrate), X_3 (CSLP), X_4 (KH_2PO_4), X_5 ($MgSO_4$), X_6 ($MnSO_4 \cdot H_2O$), X_7 ($FeSO_4 \cdot 7H_2O$).

ascent path.

Response surface methodology was used to optimize the chosen factors to enhance acetoin production based on central composite design with five coded levels. The level of each factor and the design matrix are given in Table 3. The role of each variable, their interactions, and statistical analysis to obtain predicted yields are explained by applying the following quadratic equation [36]:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (2)$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interaction effect, and X_i and X_j are input variables that influence the response variable Y.

2.5. Statistical analysis

The experimental designs and regression analysis of the experimental data were conducted with Minitab 15.0 (Minitab Inc., State College, PA, USA). All experiments were repeated three times. An analysis of variance was performed to evaluate the model. The quality of the polynomial model equation was judged statistically by the determination coefficient (R^2), and its statistical significance was determined by Fisher's test. The significance of the regression coefficients was tested using Student's t-test.

2.6. Batch and fed-batch fermentations in a 3.7-L bioreactor

To explore the behavior of acetoin accumulation, batch and fed-batch fermentations were conducted in a 3.7-L bioreactor (KLF2000 Bioengineering, Wald, Switzerland) with an initial broth volume of 2-L. The prepared seed culture was inoculated (5%, v/v) into the medium with an initial pH of 7.0. The bioreactor was operated at a temperature of 28ºC and airflow of 1.25 vvm, and agitation speed was controlled at 500, 600, 700, and 800 rpm, respectively. When the pH decreased to 6.0, it was maintained at 6.0 by automatically adding 2 M H_3PO_4 or 4 M KOH using a computer-coupled peristaltic pump. Samples were collected to determine biomass, residual sucrose, and product (acetoin and 2,3-butanediol) concentrations at desired intervals. When sucrose concentration decreased to 10 g/L in the fed-batch fermentation, sucrose solution (800 g/L) was pumped into the bioreactor to maintain residual sucrose concentration at this level.

3. Results and Discussion

3.1. PB design

In this section, a 12 experiment PB design was used to screen significant factors for seven acetoin production variables. The medium included sucrose, ammonium citrate, corn steep liquor powder (CSLP), KH_2PO_4, $MgSO_4$, $MnSO_4 \cdot H_2O$, and $FeSO_4 \cdot 7H_2O$, based on preliminary experiments and related reports [30,37,38]. Table 1 shows the PB design and corresponding acetoin production. The experimental data analysis indicated that there was a wide variation in acetoin concentrations from 23.0 to 37.6 g/L in the 12 experiments. After analysis of the regression coefficients, sucrose, ammonium citrate, CSLP, KH_2PO_4, $MnSO_4 \cdot H_2O$, and $FeSO_4 \cdot 7H_2O$ showed positive effects, whereas $MgSO_4$ showed a negative effect. The P-values of ammonium citrate, KH_2PO_4, $MgSO_4$, $MnSO_4 \cdot H_2O$, and $FeSO_4 \cdot 7H_2O$, as determined by the Students' t-test, were all > 0.05 and were considered insignificant, whereas sucrose and CSLP showed P values < 0.05 and were considered

significant.

To approach the optimum response, the fitted first-order model equation for acetoin production was obtained from the PB design experiments:

$$Y=30.4750+3.1750X_1+0.7417X_2+2.6583X_3+0.7083X_4 -0.6917X_5+0.1917X_6+0.0750X_4 \quad (3)$$

A statistical analysis of the model showed that the R^2 value was 0.9508, indicating that 95.08% of the variability in the response could be explained by the model. The value of the adjusted determination coefficient (adj. $R^2 = 0.8647$) was also very high, indicating high significance of the model.

Although sucrose and CSLP were identified as two significant factors, the optimal levels of individual factors were still unknown but were determined by the following optimization experiments.

3.2. The path of steepest ascent

Based on the model obtained using Eq. 3, the path of steepest ascent was employed to move rapidly towards the optimum response, *i.e.*, increasing the sucrose and CSLP concentrations to improve acetoin yield. From the X_1 and X_3 coefficients (3.1750, 2.6583), which were approximately equivalent (1, 0.84), the CSLP concentration (X_3) would increase 0.84 design units (1.68 g/L) if the sucrose concentration (X_1) increased 1 unit (10 g/L). The center point of the PB design was considered the path origin. The design and responses of the steepest ascent experiments are shown in Table 2. When the position where a maximum response appeared was near the optimal region, it was considered the center point for further optimization experiments. In this study, the maximum acetoin yield was obtained when sucrose and CSLP concentrations were 160 and 9.4 g/L, respectively. This point was an appropriate center point for response surface methodology.

3.3. Response surface methodology

The two significant independent variables (sucrose and CSLP) were further explored using response surface methodology. The design matrix of the variables based on central composite design and the responses of the experiments are listed in Table 3. The following second-order polynomial equations were obtained by applying multiple regression analysis:

$$Y=41.3600+1.5248X_1+0.7730X_2-3.0550X_1^2-1.7050X_2^2 +0.9500X_1X_2 \quad (4)$$

A statistical analysis of the model showed that the R^2 value was 0.9904, indicating that 99.04% of the variability in the response could be explained by the model. The adj. R^2 was 0.9835, indicating high model significance.

The effects of sucrose and CSLP on acetoin production were also determined by the three-dimensional response surface (Fig. 1). It was evident that the response surface was convex in nature, suggesting well-defined optimum conditions. It was also obvious that acetoin production had a maximum point in the studied region. When $X_1 = 0.3000$ (163 g/L, sucrose) and $X_2 = 0.3000$ (9.8 g/L, CSLP), the predicted maximum acetoin yield corresponding to these values was up to 41.7 g/L.

3.4. Validation of the optimized conditions in shaker flasks

Based on the medium optimization results, the optimum composition for acetoin production by *S. marcescens* H32 was as follows (g/L): sucrose 163, ammonium citrate 12, CSLP 9.8, KH_2PO_4 0.9, $MgSO_4$ 0.2, $MnSO_4{\cdot}H_2O$ 0.15, $FeSO_4{\cdot}7H_2O$ 0.02. Validation experiments were performed using the optimized medium to verify the predicted results. The experiments yielded an average concentration of 41.5 g/L, which was close to the model predicted value of 41.7 g/L. The correlation between the predicted and measured values of these experiments demonstrated the validity of the response model.

Table 2. Experimental design and results of the path of steepest ascent

Run	Sucrose (g/L)	CSLP (g/L)	Acetoin (g/L)
1	140	6.0	34.5
2	150	7.7	37.0
3	160	9.4	41.2
4	170	11.0	39.1
5	180	12.7	37.6

Table 3. Experimental design and results of the central composition experiments

Run	Coded variable level		Real variable level		Y (g/L)
	X_1	X_2	Sucrose (g/L)	CSLP (g/L)	
1	-1.00000	-1.00000	150	7.7	35.5
2	1.00000	-1.00000	170	7.7	36.8
3	-1.00000	1.00000	150	11.0	34.5
4	1.00000	1.00000	170	11.0	39.6
5	-1.41421	0.00000	145.9	9.4	33.2
6	1.41421	0.00000	174.1	9.4	37.3
7	0.00000	-1.41421	160	7.0	36.4
8	0.00000	1.41421	160	11.7	39.5
9	0.00000	0.00000	160	9.4	41.2
10	0.00000	0.00000	160	9.4	41.5
11	0.00000	0.00000	160	9.4	41.2
12	0.00000	0.00000	160	9.4	41.3
13	0.00000	0.00000	160	9.4	41.6

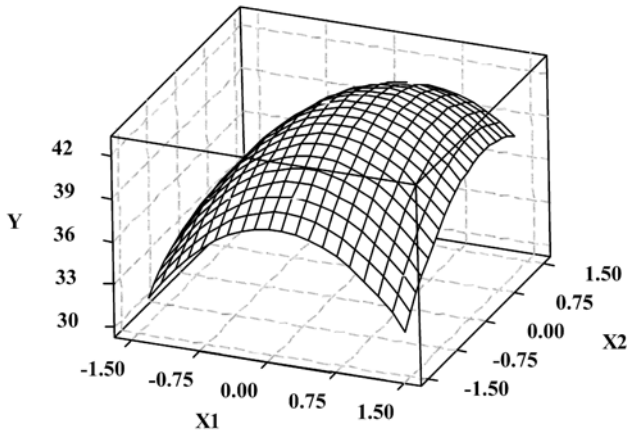

Fig. 1. Response surface graph of the mutual effects of sucrose (X_1) and corn steep liquor powder (CSLP) (X_2) on acetoin production (Y).

3.5. Batch fermentations with different agitation speeds

The effects of different agitation speeds (500, 600, 700, and 800 rpm) on acetoin fermentation were investigated. The results indicated that agitation speed played a vital role in acetoin production. As shown in Figs. 2A, 2B, 2C, and 2D and Table 4, the relatively higher final acetoin concentration of up to 45.1 g/L was obtained at an agitation speed of 600 rpm. When the agitation speed was higher or lower, the final acetoin concentration decreased (38.5 g/L at 500 rpm, 40.7 g/L at 700 rpm, and 33.5 g/L at 800 rpm), indicating that either lower agitation speed (500 rpm) or higher agitation speed (700 and 800 rpm) did not benefit acetoin production.

Additionally, the cells grew rapidly during batch fermentations after an adaptation period of 2 h. As cell density continued to increase, oxygen demand also increased and DO fell rapidly. Acetoin and 2,3-butanediol started to accumulate when oxygen became limited. The reason might be that during aerobic growth, the NADH generated from glycolysis was oxidized with oxygen *via* the respiratory chain. When oxygen became short in supply, the respiratory chain could not effectively regenerate the excess reducing power. Therefore, the acetoin and 2,3-butanediol pathway was activated to oxidize NADH and

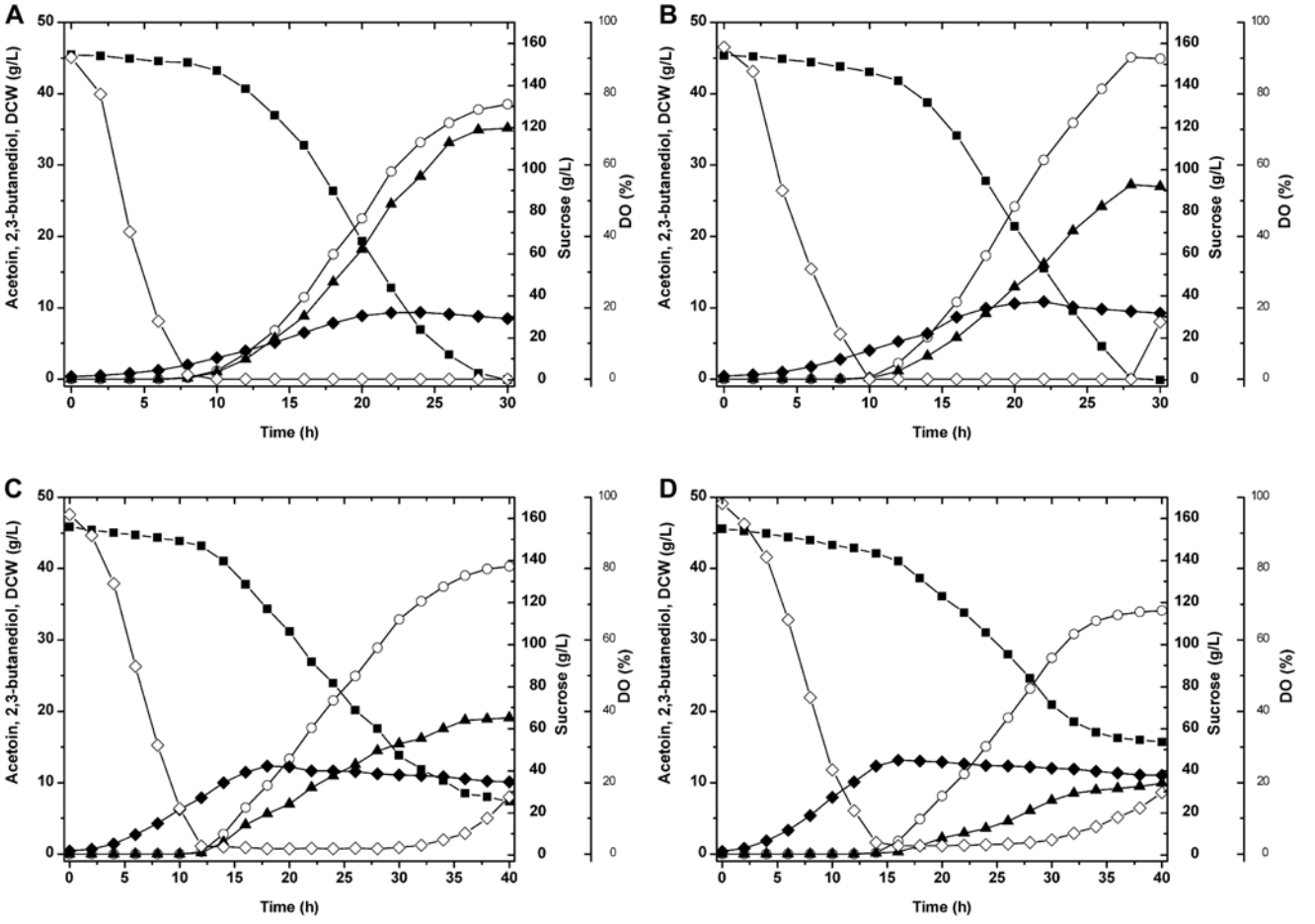

Fig. 2. Time course of acetoin production by *Serratia marcescens* H32 in batch fermentations at different agitation speeds: (A) 500 rpm; (B) 600 rpm; (C) 700 rpm; and (D) 800 rpm. Symbols: acetoin (○), 2,3-butanediol (▲), DCW (◆), sucrose (■), dissolved oxygen (DO) (◇).

Table 4. Comparison of parameters in batch fermentation of acetoin at different agitation speed control strategies

Parameters	Agitation speed (rpm)					
	500	600	700	800	700 ~ 600[a]	800 ~ 600[b]
DCW (g/L)	9.3	11.1	12.3	13.1	10.8	10.6
Acetoin (g/L)	38.5	45.1	40.7	34.1	44.9	43.6
2,3-butanediol (g/L)	35.2	27.2	19.1	9.9	26.8	25.5
Sucrose consumption rate (g/L/h)	5.16	5.54	3.38	2.53	5.96	5.52
Acetoin productivity (g/L/h)	1.28	1.61	1.02	0.85	1.73	1.56

[a]Agitation speed was kept at 700 rpm before 8 h, and then switched to 600 rpm.
[b]Agitation speed was kept at 800 rpm before 7 h, and then switched to 600 rpm.

maintain the NAD^+/NADH balance *in vivo* [39].

Furthermore, cell growth was favored with higher agitation speeds (700 and 800 rpm). However, sucrose consumption rates were relatively lower under these conditions, which lead to lower acetoin production. This might be because under higher oxygen supply conditions more NADH is oxidized *via* the respiratory chain and more ATP is produced. An increased ATP charge decreases glycolysis rate, as the key enzyme of glycolysis, phosphofructokinase, is inhibited by elevated ATP levels [40]. It took *S. marcescens* H32 10 h to reach the DCW value of 4 g/L under an agitation speed of 600 rpm, at which point the DO fell to near zero, and the acetoin pathway was activated. Only 7 or 8 h were needed for *S. marcescens* H32 to reach this DCW point under an agitation speed of 800 or 700 rpm, respectively. Cell activity was relatively high at the initial stage of fermentation, and oxygen demand was mainly determined by cell density. Thus, agitation speed was first controlled at a higher value to achieve a faster cell growth rate and then switched to 600 rpm to reduce the DO. The acetoin formation pathway might be activated earlier than that under single agitation speed control conditions.

3.6. Two-stage agitation speed control strategy for acetoin fermentation

A two-stage agitation speed control strategy was proposed based on the analysis described above. The agitation speed was controlled at 700 or 800 rpm in the first 8 or 7 h and then switched to 600 rpm. The fermentation results of the two-stage agitation speed control strategy are shown in Fig. 3 and Table 4. Acetoin accumulation time was earlier using the two-stage agitation speed control strategy than that using the single-agitation speed control strategy.

When agitation was first controlled at 700 rpm, and then changed to 600 rpm, the maximum acetoin concentration reached 44.9 g/L with productivity of 1.73 g/L/h. Acetoin production was similar to the single-agitation speed of 600 rpm (45.1 g/L) but productivity was 7.5% higher (1.61 g/L/h). However, when agitation speed was first controlled at 800 rpm and then changed to 600 rpm, the maximum acetoin concentration was only 43.6 g/L. Although acetoin accumulation time was the earliest of all strategies, acetoin productivity was only 1.56 g/L/h. The reason might be that the key enzyme in the acetoin formation pathway, α-acetolactate synthase, is inactivated under high oxygen

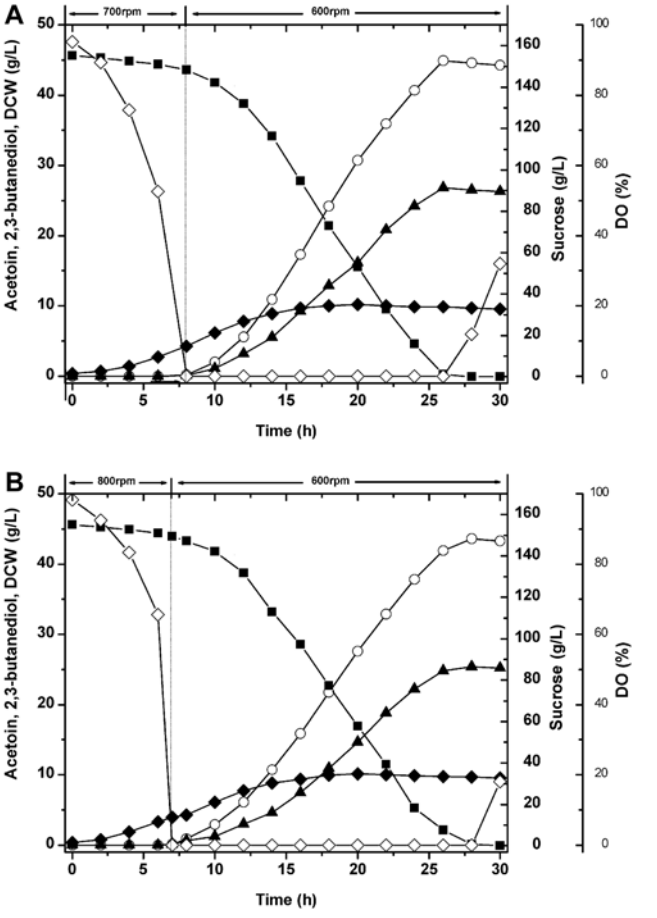

Fig. 3. Time course of acetoin production during batch fermentation by *Serratia marcescens* H32 using the two-stage agitation speed control strategy: (A) 700 rpm for the first 8 h then switched to 600 rpm; (B) 800 rpm for the first 7 h then switched to 600 rpm. Symbols: acetoin (○), 2,3-butanediol (▲), DCW (◆), sucrose (■), dissolved oxygen (DO) (◇).

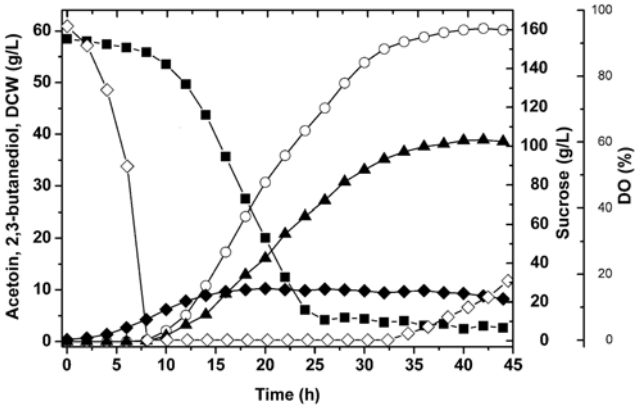

Fig. 4. Time course of acetoin production in fed-batch fermentation by *Serratia marcescens* H32. Symbols: acetoin (○), 2,3-butanediol (▲), DCW (◆), sucrose (■), dissolved oxygen (DO) (◇).

supply conditions [41,42], and the dramatic changes in agitation speed had harmful effects on cell activity.

3.7. Fed-batch fermentation with the two-stage agitation speed control strategy

During the two-stage agitation speed control fermentation, it was observed that acetoin concentration continued to increase when sucrose was exhausted. Thus, fed-batch fermentation by feeding a sucrose solution was carried out. The agitation speed was initially controlled at 700 rpm and then changed to 600 rpm after 8 h. As shown in Fig. 4, the highest acetoin concentration reached 60.5 g/L, and acetoin productivity was 1.44 g/L/h after 42 h of fermentation. The main byproduct, 2,3-butanediol, which could be used widely in the chemical industry, also accumulated up to 38.9 g/L.

Compared with 2,3-butanediol production, which has achieved great success, much less attention has been paid to acetoin production. Fortunately, more studies have been focusing on acetoin fermentation over the last few years, and relatively high acetoin concentrations of up to 42.2, 46.9, and 63 g/L, with productivities of 0.32, 0.73, and 1.05 g/L/h have been obtained [15-17]. Different from the former studies, most of which employed batch fermentation to produce acetoin, this study applied a fed-batch operation to perform the fermentation process. The fed-batch fermentation effectively avoided substrate inhibition and made full use of cell fermentative activity. Using the fed-batch fermentation, a relatively high level of acetoin production (60.5 g/L) and productivity (1.44 g/L/h) were attained.

4. Conclusion

A suitable medium component for acetoin production by *S. marcescens* H32 was developed using a sequential statistical experimental design. Based on the optimized medium, a two-stage agitation speed control strategy was established in a 3.7-L bioreactor. Fed-batch fermentation was ultimately performed, and a maximum acetoin concentration of 60.5 g/L with productivity of 1.44 g/L/h was achieved. This study has provided a new way to industrially ferment acetoin.

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