

## Utilization of Sweet Sorghum Juice for Efficient 2,3-Butanediol Production by *Serratia marcescens* H30

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Sweet sorghum juice (SSJ) is considered a good carbon source for biorefinery due to its low price and high fermentable-sugar content. In this study, 2,3-butanediol (2,3-BD) production from SSJ by *Serratia marcescens* H30 was investigated. First, the medium compositions including the contents of SSJ, nitrogen source, and mineral salts were optimized in conical flasks using a single factor and orthogonal design method. Under the optimal conditions, the 2,3-BD concentration reached up to 33.40 g/L. Then the optimized medium was used to perform fermentative experiments in a 5-L bioreactor. In batch experiments, the effects of various agitation speeds on 2,3-BD production were compared. Based on batch process results, an efficient two-stage fermentative control strategy was developed, where the agitation speed was maintained at 300 rpm in the first 12 h and subsequently switched to 200 rpm. About 43.32 g/L 2,3-BD was obtained by using this strategy. Finally, fed-batch fermentation was conducted through feeding the concentrated SSJ and a maximum 2,3-BD concentration of 109.44 g/L with the productivity of 1.40 g/L·h; a yield of 83.02% was achieved. The results showed that SSJ could be used as an economical substrate for efficient 2,3-BD production by *S. marcescens* H30.

*Keywords:* 2,3-Butanediol; Sweet sorghum juice; *Serratia marcescens*; Medium optimization; Fed-batch

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### INTRODUCTION

With the development of the bio-refinery industry, many industrial chemicals can now be produced from renewable resources. Microbial production of 2,3-butanediol (2,3-BD) is one such example. As an important platform intermediate, 2,3-BD could be used to produce numerous other chemical compounds such as methylethyl ketone, butadiene, and butanone (Celinska and Grajek 2009; Ji *et al.* 2011; Syu 2011; Adlakha and Yazdani 2015). It can also be used as a fuel additive due to its combustion heat energy of 27.2 kJ/g (Li *et al.* 2014a; Yang *et al.* 2015). Furthermore, 2,3-BD can be readily dehydrogenated into acetoin (AC) and diacetyl (DA) compounds, which are used as flavor enhancers in dairy products (Guo *et al.* 2016; Zhang *et al.* 2016). Some microbial fermentation processes have been developed to enhance the 2,3-BD production in recent years. High 2,3-BD concentrations and productivities have been achieved from pure carbon sources by use of several microbial strains such as *Klebsiella pneumoniae*, *Serratia marcescens*, and *Bacillus licheniformis* (Ma *et al.* 2009; Zhang *et al.* 2010a; Jurchescu *et al.* 2013).

However, biological large-scale production of 2,3-BD is still in its early stage, mainly due to its manufacturing cost (Yang *et al.* 2015). In microbial fermentations, 2,3-BD is produced mainly from the conversion of the substrates, which accounts for more than half of the total production cost and would seriously influence the 2,3-BD production economy (Koutinas *et al.* 2016). Therefore, efficient 2,3-BD production from the most abundant, cheap, and renewable source of sugar substrates by microbial fermentation is desired.

To decrease the cost, some crude renewable resources including corncob molasses, sugarcane molasses, crude glycerol, starch, seaweed hydrolysate, raw inulin extract from Jerusalem artichoke, spirit-based distillers' grain, and lignocellulosic hydrolysates have been investigated for 2,3-BD production by using various microbial strains (Grover *et al.* 1990; Afschar *et al.* 1991; Cao *et al.* 1997; Cheng *et al.* 2010; Gao *et al.* 2010; Petrov and Petrova 2010; Wang *et al.* 2010; Jiang *et al.* 2012; Metsoviti *et al.* 2012; Jung *et al.* 2013; Suman *et al.* 2013; Huang *et al.* 2013; Li *et al.* 2014b; Zhang *et al.* 2014a; Yang *et al.* 2015; Li *et al.* 2016).

However, the environmentally unfriendly pre-treated processes or low product yields have limited the application of these sources. In comparison with starchy or lignocellulosic biomass feedstocks, the juice from fresh stalk of energy crop containing high concentration of fermentable sugar has been considered to be an interesting alternative choice (Cai *et al.* 2016; Maw *et al.* 2016). Sweet sorghum has been recognized as a promising energy crop since it can be readily cultivated in tropical climate areas with high photosynthetic efficiency and wide adaptability to different environmental conditions (Ratnavathi *et al.* 2011; Dutra *et al.* 2013). Furthermore, its stalks contain highly fermentable sugars (sucrose, glucose, and fructose), nitrogen, vitamins, amino acids, inorganic salts, and other stimulants (Laopaiboon *et al.* 2009; Ratnavathi *et al.* 2011; Ariyajaroenwong *et al.* 2012; Deesuth *et al.* 2016). So the sweet sorghum juice (SSJ) has been considered an important source for valuable chemicals and biofuels production (Deesuth *et al.* 2015, 2016; Sirisantimethakom *et al.* 2016). To date, 2,3-BD production from SSJ through microbial fermentation has not been reported.

*S. marcescens* H30 is a promising industrial strain for 2,3-BD production due to high yield, better resistance to bacterial contamination, broad substrate spectrum, and cultural adaptability (Zhang *et al.* 2010a; Sun *et al.* 2012; Rao *et al.* 2012). Meanwhile, it can produce high purity of *meso*-2,3-BD, which has been found to occupy over 98% weight fraction (Zhang *et al.* 2010a; Li *et al.* 2014c). Previous studies by the authors have showed that high 2,3-BD concentration (152 g/L) could be achieved by this strain using pure sucrose as the substrate (Zhang *et al.* 2010b).

The present work aimed to investigate the feasibility of 2,3-BD production from the SSJ as the substrate using *S. marcescens* H30. Firstly, the SSJ as the sole raw material for 2,3-BD production by *S. marcescens* H30 was evaluated. The results showed that low 2,3-BD concentration could be obtained. Subsequently, some exogenous factors such as nitrogen sources and inorganic salts were supplemented into the medium to improve 2,3-BD production by *S. marcescens* using an orthogonal array design method. A relatively high product yield could be achieved under the optimal medium. Ultimately, batch and fed-batch experiments in a 5-L bioreactor were investigated, and the accumulation of 2,3-BD was enhanced substantially.

## EXPERIMENTAL

### Materials

Sweet sorghum stalk was kindly provided by Institute of Energy Crop, Fujian Agriculture and Forestry University (China), and was harvested on December 2015 from Qingkou experimental field in Fuzhou city, Fujian Province. After cutting heads and removal of leaves, the stalks were placed in the shade for one week, and subsequently squeezed by a sugarcane extractor to obtain the fresh juice. The obtained juice after filtration and centrifugation was stored at -20 °C to minimize microbial growth before use.

### Methods

#### *Microorganism and seed preparation*

The strain of *S. marcescens* H30 was streaked on LB agar slants and incubated at 30 °C for 16 h. The slants were maintained at 4 °C for further fermentative experiments. The seed preparation was conducted at 30 °C with shaking at 180 rpm for 12 h in a seed medium containing 10 g/L of glucose, 2 g/L of peptone, 1 g/L of yeast extract, 6 g/L of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 g/L of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L of NaCl, and 0.5 g/L of MgSO<sub>4</sub> at pH 7.2 (Zhang *et al.* 2010a).

#### *2,3-BD fermentation using the SSJ as the sole medium*

The initial fermentative medium containing the SSJ as the sole raw material was used to evaluate 2,3-BD production by *S. marcescens* H30. The juice concentrations were varied as 25%, 50%, 75%, and 100% by dilution with different volumes of deionized water and were tested in this study. The fermentation experiments were performed in 250 mL shake flasks containing 50 mL fresh medium for 36 h at 150 rpm and 30 °C in triplicate.

#### *Effect of nitrogen sources and inorganic salts on 2,3-BD production*

Five nitrogen sources including corn steep liquor, soybean meal, yeast extract, peptone, and ammonium sulfate at the concentration of 5 g/L were chosen to evaluate their effects on 2,3-BD production and the growth of *S. marcescens* H30. The optimum nitrogen source was used to carry out further experiments. According to the results from previous studies (Zhang *et al.* 2010a; Sun *et al.* 2012), several inorganic salts (sodium acetate 0-10 g/L, KH<sub>2</sub>PO<sub>4</sub> 0-1 g/L, MgSO<sub>4</sub> 0-0.5 g/L, MnSO<sub>4</sub> 0-0.5 g/L, FeSO<sub>4</sub> 0-0.5 g/L, and ZnCl<sub>2</sub> 0-0.5 g/L) were used to investigate the effects on 2,3-BD production by *S. marcescens* H30. All the experiments using various inorganic salts were carried out in 50 mL fresh medium containing the SSJ content of 75% and 5 g/L yeast extract for 36 h at 150 rpm and 30 °C in triplicate.

#### *Optimization of medium compositions*

Based on the above results of single factor experiments, the orthogonal array design (OAD) method was employed to test typical pairs of combinations of factors and to identify their best combination. OAD, also known as the Taguchi method, incorporates the advantages of the simplex method and factorial design. OAD can efficiently reduce the number of tests and achieve the optimum value. It can also arrange different factors for effective optimization of the experimental conditions (Yang *et al.* 2011; Khongsay *et al.* 2012). The major variables influencing 2,3-BD production were the contents of SSJ,

yeast extract, sodium acetate,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ , and  $\text{ZnCl}_2$ . Therefore, a  $L_{18}$  ( $3^7$ ) orthogonal array was used to design the experiments as shown in Table 2. The levels of each factor were chosen according to the above investigated results of single factor experiments. All the experimental combinations in Table 2 were performed at 30 °C and 150 rpm for 36 h in triplicate.

#### *Batch and fed-batch fermentations*

Batch and fed-batch fermentations were conducted in a 5-L bioreactor (BaoXingBiao, Shanghai) with an initial broth volume of 3 L. The prepared seed culture was inoculated (5%, v/v) into the optimized SSJ medium with an initial pH of 7.0 (Zhang *et al.*, 2010). The bioreactor was operated at 30 °C with an aeration rate of 0.5 vvm (air volume per culture volume per min). To explore the effects of agitation speeds of 100, 200, and 300 rpm on 2,3-BD production by *S. marcescens* H30 various reactions were investigated during batch fermentation process. Based on the optimal medium and suitable operation conditions, fed-batch fermentation was conducted by an interim feeding strategy. When the sugar in the fermentation broth was decreased to 10 g/L, the concentrated SSJ solution (150 mL, about 800 g/L) was supplemented into the fermentation broth each time. The concentrated SSJ solution was prepared by rotary evaporation (4 L raw SSJ was concentrated to about 800 mL solution). The pH value was controlled at 6.0 by automatic addition of 4 M NaOH solution using a computer coupled peristaltic pump when it decreased to 6.0. During the course of the entire batch and fed-batch fermentation, the samples were collected to determine biomass, residual sugar, and product concentrations at desired intervals.

#### *Analytical methods*

The SSJ compositions including sugar, total nitrogen, and metal ions were analyzed by Fujian Inspection and Research Institute (China) of product quality (Laopaiboon *et al.* 2009; Cai *et al.* 2016). The biomass concentration was determined by the optical density at the wavelength of 600 ( $\text{OD}_{600}$ ) in a spectrophotometer (UV-1800, Mapada, China) and correlated with dry cell weight (DCW) as described previously (Zhang *et al.* 2010a). The linear equation is  $\text{DCW (g/L)} = 0.422 * \text{OD}_{600} - 0.0363$ .

The residual sugars including sucrose, glucose, and fructose were determined by a high performance liquid chromatography (Agilent 1100, USA) equipped with Agilent Zorbax carbohydrate column (Cai *et al.* 2016). The product 2,3-BD and its precursor AC were quantified *via* gas chromatography (Agilent GC9860, FID detector, DB-5 column). The operation conditions were as follows:  $\text{N}_2$  was used as carrier gas at a flow rate of 1.5 mL/min; both the injector and detector temperatures were kept at 215 and 245 °C, respectively; the initial column temperature was controlled at 50 °C for 1.5 min, subsequently raised to 180 °C at a rate of 25 °C/min (Sun *et al.* 2012). The injection volume was 1  $\mu\text{L}$  and the split ratio was 1:10. External standard method was used to calculate the concentrations of the products in the samples.

The 2,3-BD percentage yield (%) = 2,3-BD yield (g/L)/Theoretical yield (g/L). Theoretically, one mole of glucose or fructose can generate one mole of 2,3-BD, whereas two moles of 2,3-BD can be produced from one mole of sucrose. Minitab 15 software was used for data analysis.

## RESULTS AND DISCUSSION

### Chemical Composition of the SSJ

The chemical compositions of SSJ were analyzed and are shown in Table 1. The raw SSJ solution contained a high concentration of fermentable sugar, which mainly consisted of sucrose (136.0 g/L), glucose (11.9 g/L), and fructose (9.8 g/L). Sucrose occupied a weight fraction of over 86% of the total fermentable sugar. As reported previously, the strain of *S. marcescens* H30 could efficiently utilize high concentration sucrose for 2,3-BD production achieving high yield (Zhang *et al.* 2010a). Therefore, the SSJ with high sugar concentration has the potential for 2,3-BD production by this strain. Beside fermentable sugar, the raw SSJ also contained many minerals and trace elements as well as nitrogen source. Among the analyzed minerals, potassium had the highest content, at the level of 7460.0 mg·kg<sup>-1</sup>. The total nitrogen, calcium, and magnesium contents were 780.0, 460.0, and 217.0 mg·kg<sup>-1</sup>, respectively. These nutrients required for the strain growth and 2,3-BD production partially reduces the 2,3-BD production cost.

**Table 1.** Composition of SSJ

Carbon sources	Concentration (g/L)	Nutrients	Concentrations (mg/kg)
Sucrose	136.0	P	0.4
Glucose	11.9	Cu	0.5
Fructose	9.8	Zn	8.3
		Fe	2.2
		Mg	217.0
		Mn	4.5
		K	7460.0
		Na	24.9
		Ca	460.0
		N	780.0

### Direct Production of 2,3-BD from SSJ

The SSJ containing a large amount of fermentable sugars with many minerals and trace elements might be used as the sole medium to produce 2,3-BD by microbial fermentation. Hence, the growth of *S. marcescens* H30 and its 2,3-BD production with different concentrations of SSJ were investigated. The results are shown in Fig. 1. The results showed that *S. marcescens* H30 could grow efficiently on the SSJ medium and the biomass concentration was increased with the SSJ contents from 25% to 100%. The maximum biomass concentration of 2.72 g/L was obtained at 36 h using the SSJ content of 100%, which was remarkably less than that of 8.40 g/L using the optimized medium containing pure sucrose in our previous study (Zhang *et al.* 2010a). The explanation for these results might include substrate inhibition and nutrients inadequacy for the growth of *S. marcescens* H30. Meanwhile, the low biomass concentration also resulted in the decrease of 2,3-BD production and the prolongation of fermentation time, as shown in Fig. 1. Ultimately, the SSJ contents of 75% and 100% produced 14.08 g/L and 16.19 g/L of 2,3-BD respectively at 36 h by *S. marcescens* H30.

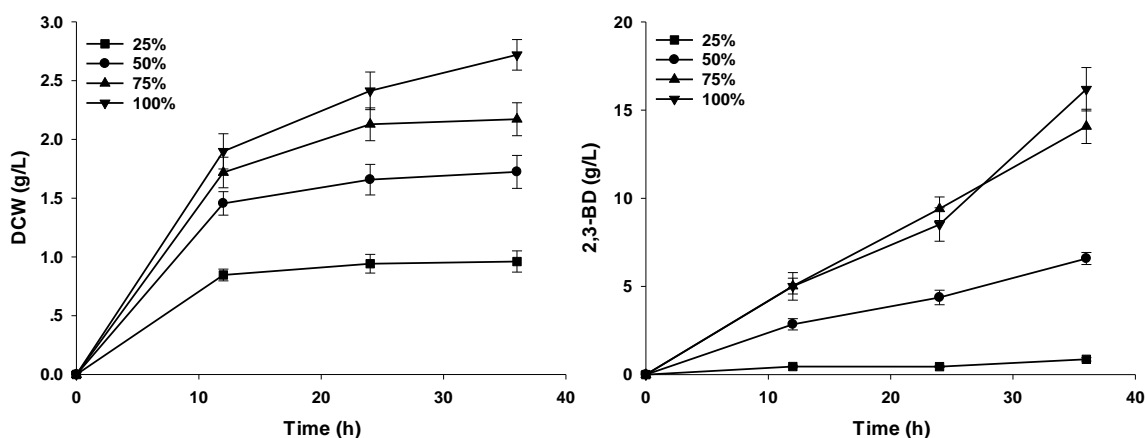


Fig. 1. *S. marcescens* H30 growth and 2,3-BD production using various concentrations of SSJ

### Effects of Nitrogen Sources on 2,3-BD Fermentation

The above results indicated that the nutrients present in the SSJ medium were inadequate for 2,3-BD production by *S. marcescens* H30, despite the fact that the SSJ medium contained a large amount of fermentable sugar. Previous studies using *Saccharomyces cerevisiae* have shown that an extra nitrogen source supplemented into the SSJ medium could effectively improve ethanol production (Laopaiboon *et al.* 2009; Deeth *et al.* 2012). To explore a suitable nitrogen source, the effect of different nitrogen sources at the concentration of 5 g/L on the strain growth and 2,3-BD production was investigated. As shown in Fig. 2, both the biomass and 2,3-BD production could be improved when five different nitrogen sources were supplemented into the SSJ medium.

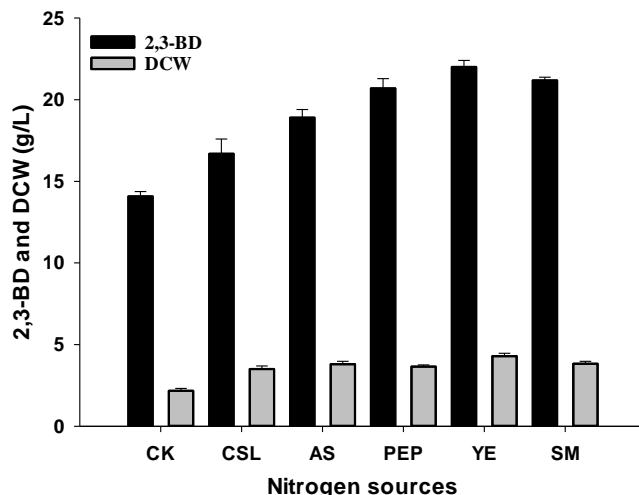


Fig. 2. Effect of different nitrogen sources at the concentration of 5 g/L on *S. marcescens* H30 growth and 2,3-BD production: CK: control; CSL: corn steep liquor; AS: ammonium sulfate; PEP: peptone; YE: yeast extract; SM: soybean meal

ANOVA analysis showed that nitrogen source supplementation obviously promoted the strain growth and 2,3-BD production ( $P$ -value < 0.001). Among the analyzed nitrogen sources, yeast extract showed the highest biomass and 2,3-BD yield during the fermentation process. About 4.29 g/L dry cell weight (DCW) and 22.01 g/L of

2,3-BD were achieved by *S. marcescens* H30 at 36 h in the presence of SSJ (75%) and yeast extract (5 g/L). These results indicated that the supplementation of nitrogen sources into the SSJ medium favored the strain growth and 2,3-BD production.

### Effects of Inorganic Salts on 2,3-BD Fermentation

Our previous studies showed that several inorganic salts such as sodium acetate,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ ,  $\text{FeSO}_4$ , and  $\text{ZnCl}_2$  exhibited positive effects on 2,3-BD production by *S. marcescens* H30 (Zhang *et al.* 2010a; Sun *et al.* 2012). Sodium acetate as an inducer could increase the transcription level of 2,3-BD pathway in *S. marcescens* H30 and other 2,3-BD producing microbial strains (Zeng *et al.* 1990; Ji *et al.* 2011; Rao *et al.* 2012), and further result in 2,3-BD accumulation during the fermentation process. The use of  $\text{KH}_2\text{PO}_4$  could provide a phosphorus source for the growth of 2,3-BD producing strains (Zhang *et al.* 2010a; Sun *et al.* 2012). In addition, it has been reported that metal ions including  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Zn}^{2+}$  were very important for the strain growth and 2,3-BD production by activating the activities of the related enzymes (Zhang *et al.* 2014). Therefore, these inorganic salts were chosen to investigate their effects on 2,3-BD production by *S. marcescens* H30. The results indicated that all the listed inorganic salts except  $\text{FeSO}_4$  could improve 2,3-BD production during the fermentation process (Fig. 3).

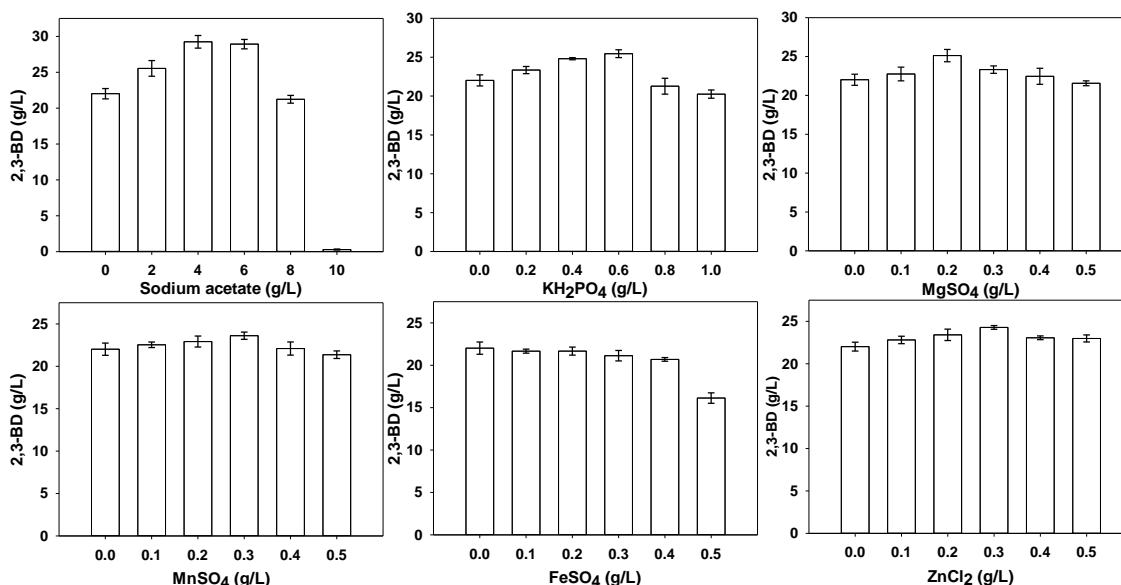


Fig. 3. Effects of different metal ions on 2,3-BD production by *S. marcescens* H30

Sodium acetate and  $\text{KH}_2\text{PO}_4$  showed significantly positive effect for 2,3-BD production ( $P$ -value < 0.005). Especially sodium acetate exhibited an obvious activation effect for 2,3-BD production and led to a maximum increase by 32.85% at the concentration of 4 g/L. Whereas  $\text{FeSO}_4$  showed slight inhibition during the 2,3-BD fermentation process. A possible explanation is that  $\text{Fe}^{2+}$  influenced the conversion from acetoin to 2,3-BD. The authors' previous study have showed that the activity of 2,3-butanediol dehydrogenase (BDH) responsible for the conversion of AC to 2,3-BD in *S. marcescens* H30 could be strongly inhibited by  $\text{Fe}^{2+}$ , while  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  ions could efficiently activate and enhance the BDH catalytic activity from acetoin to 2,3-BD

(Zhang *et al.* 2014b). To obtain their optimum concentrations, these positive nutrients on 2,3-BD production were used to carry out further optimization experiments.

### Orthogonal Experimental Design of Nutrient Supplementation

An orthogonal design  $L_{18}(3^7)$  was employed to investigate the effect of nutrient supplement dosages of SSJ, yeast extract, sodium acetate,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ , and  $\text{ZnCl}_2$  on 2,3-BD production by *S. marcescens* H30. Eighteen groups were used to identify the best combination of these seven variables. Each variable was set at three levels according to the results of single factor experiments, as shown in Table 2.

**Table 2.** Experimental Design and Results of the Orthogonal Array Design form  $[L_{18}(3^7)]$  for 2,3-BD Production

Experimental number	Operating factors and their levels (g/L)							2,3-BD (g/L)
	SSJ	Yeast extract	Sodium acetate	$\text{KH}_2\text{PO}_4$	$\text{MgSO}_4$	$\text{MnSO}_4$	$\text{ZnCl}_2$	
1	1 (80)	1 (5)	1 (2)	1 (0.2)	1 (0.1)	1 (0.1)	1 (0.1)	23.97 ± 0.68
2	1	2 (10)	2 (4)	2 (0.4)	2 (0.2)	2 (0.2)	2 (0.2)	24.92 ± 0.65
3	1	3 (15)	3 (6)	3 (0.6)	3 (0.3)	3 (0.3)	3 (0.3)	25.29 ± 0.21
4	2 (96)	1	1	2	2	3	3	24.18 ± 0.58
5	2	2	2	3	3	1	1	26.78 ± 0.55
6	2	3	3	1	1	2	2	30.72 ± 0.19
7	3 (112)	1	2	1	3	2	3	25.70 ± 0.49
8	3	2	3	2	1	3	1	29.04 ± 0.68
9	3	3	1	3	2	1	2	22.91 ± 0.64
10	1	1	3	3	2	2	1	18.14 ± 0.61
11	1	2	1	1	3	3	2	25.62 ± 0.44
12	1	3	2	2	1	1	3	24.86 ± 0.61
13	2	1	2	3	1	3	2	26.49 ± 0.34
14	2	2	3	1	2	1	3	27.69 ± 0.48
15	2	3	1	2	3	2	1	24.66 ± 0.24
16	3	1	3	2	3	1	2	25.90 ± 0.64
17	3	2	1	3	1	2	3	22.83 ± 0.59
18	3	3	2	1	2	3	1	27.08 ± 0.76



The fermentation experiments for each group were carried out in triplicate, and the concentrations of 2,3-BD in the samples were determined using the GC system. The experimental results presented in Table 2 indicated that there was a wide variation of 2,3-BD concentration from 18.14 g/L to 30.72 g/L in the eighteen group experiments, which suggested that the different combinations of these seven variables had remarkable effects on 2,3-BD production by *S. marcescens* H30. Furthermore, the range analysis was provided in Table 3.  $K_1$ ,  $K_2$ , and  $K_3$  represent the average values of the data at each level in the orthogonal design experiment. The optimal level of each variable could be observed by comparing the value of  $K_i$  ( $i = 1, 2, \text{ and } 3$ ).

According to the results of range analysis, the order of influence on 2,3-BD production was  $\text{KH}_2\text{PO}_4 > \text{SSJ} > \text{MgSO}_4 > \text{Sodium acetate} > \text{Yeast extract} > \text{MnSO}_4 > \text{ZnCl}_2$ . The significance of each variable is given in Table 4. Based on the principle of ‘the larger the better’, the optimum combination of SSJ, yeast extract, sodium acetate,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{MnSO}_4$ , and  $\text{ZnCl}_2$  should be 96, 10, 6, 0.2, 0.1, 0.3, and 0.2 g/L, respectively. The verification experiment under the optimal combination was performed in flask in triplicate test and 33.40 g/L of 2,3-BD by *S. marcescens* H30 could be obtained at 36 h.

**Table 3.** Range Analysis for the  $L_{18}(3^7)$  Orthogonal Array Experiment

K value <sup>a</sup>	2,3-BD concentration (g/L)						
	SSJ	Yeast extract	Sodium acetate	$\text{KH}_2\text{PO}_4$	$\text{MgSO}_4$	$\text{MnSO}_4$	$\text{ZnCl}_2$
$K_1$	23.801	24.063	24.029	26.796	26.319	25.351	24.946
$K_2$	26.753	26.148	25.970	25.595	24.153	24.496	26.094
$K_3$	25.577	25.920	26.131	23.740	25.659	26.238	25.091
Range	2.952	2.085	2.102	3.056	2.166	1.787	1.149

<sup>a</sup>  $K_1$ ,  $K_2$ , and  $K_3$  represented the average values of the data at each level in the orthogonal design experiment.  $R$  is estimated by the difference between the highest and the lowest of the average scores, i.e.,  $K_{\max} - K_{\min}$

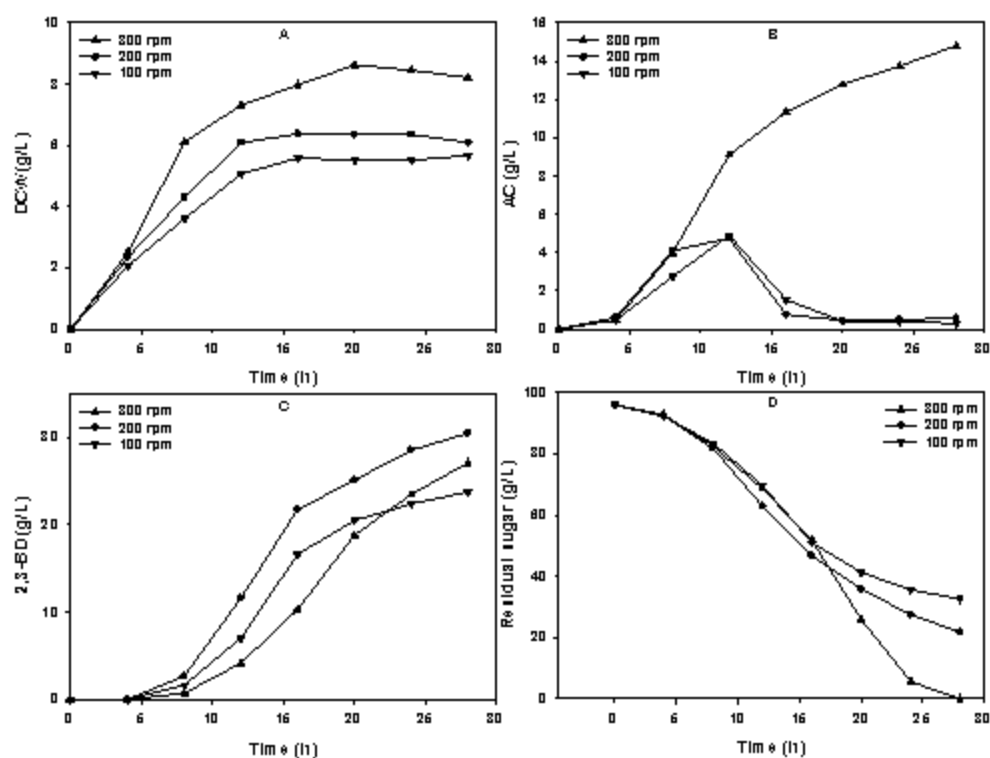
**Table 4.** ANOVA Analysis for the  $L_{18}(3^7)$  Orthogonal Array Experiment

Factors	2,3-BD				
	DF	SS	SM	F-value	P-value
SSJ	2	26.527	13.263	5.64	0.096
Yeast extract	2	15.678	7.839	3.33	0.173
Sodium acetate	2	16.437	8.219	3.49	0.165
$\text{KH}_2\text{PO}_4$	2	28.452	14.226	6.04	0.089
$\text{MgSO}_4$	2	14.776	7.388	3.14	0.184
$\text{MnSO}_4$	2	9.600	4.800	2.04	0.276
$\text{ZnCl}_2$	2	4.687	2.344	1.00	0.466
Error	3	7.061	2.354		
Total	17	123.217			

### Batch Fermentations with Various Agitation Speeds

During the fermentation process, oxygen supply was shown to be an important factor for 2,3-BD production in previous studies (Zeng *et al.* 1994; Zhang *et al.* 2010a; Ji *et al.* 2011; Sun *et al.* 2012). Therefore, it is necessary for the effect of oxygen supply on 2,3-BD production by *S. marcescens* H30 under the optimized medium to be investigated. However, the dissolved oxygen (DO) was found to rapidly decrease to zero when the batch fermentation was carried out in 5-L bioreactor.

To explore a suitable oxygen supply condition, the effects of various agitation speeds (100, 200, and 300 rpm) on 2,3-BD fermentation by *S. marcescens* H30 were investigated in a 5-L bioreactor. The concentrations of DCW, AC, 2,3-BD, and residual sugar were determined every 4 h until the end of fermentation. The results indicated that the agitation speed had a vital influence on 2,3-BD production in the batch fermentation (Fig. 4). As shown in Fig. 4C, a maximum 2,3-BD concentration of up to 30.45 g/L could be achieved at the agitation speed of 200 rpm using the optimized SSJ medium. In contrast, the final 2,3-BD concentrations at 100 and 300 rpm were 23.76 g/L and 27.06 g/L, respectively. It was demonstrated that lower agitation speed (100 rpm) or higher agitation speed (300 rpm) did not favor the 2,3-BD production. Therefore, microbial 2,3-BD production should belong to a microaerobic type fermentation process.



**Fig. 4.** Time-course of DCW, AC, 2,3-BD, and sugar consumption evolution in batch fermentation by *S. marcescens* H30 at various agitation speeds: (A) DCW; (B) AC; (C) 2,3-BD; and (D) Sugar consumption

Furthermore, the cell growth, AC, and sugar consumptions were also determined during the fermentation process. The cell growths of three different agitation speed conditions appeared similar evolution in the first 4 h due to adequate oxygen supply. Thereafter, the cell density continued to increase but different growth rates could be observed among the three fermentation conditions. A maximum DCW of 8.62 g/L could be obtained at 20 h when the agitation speed was controlled at 300 rpm, whereas the agitation speed of 100 and 200 rpm led to 6.37 g/L and 5.58 g/L DCW, respectively (Fig. 4A). These results showed that high agitation speed improved the oxygen transfer to the cell in the fermentation broth and promoted the cell growth of *S. marcescens* H30. The product 2,3-BD and its precursor AC started to accumulate after 4 h. Rapid accumulation of AC during the batch fermentation process could be observed at the agitation speed of

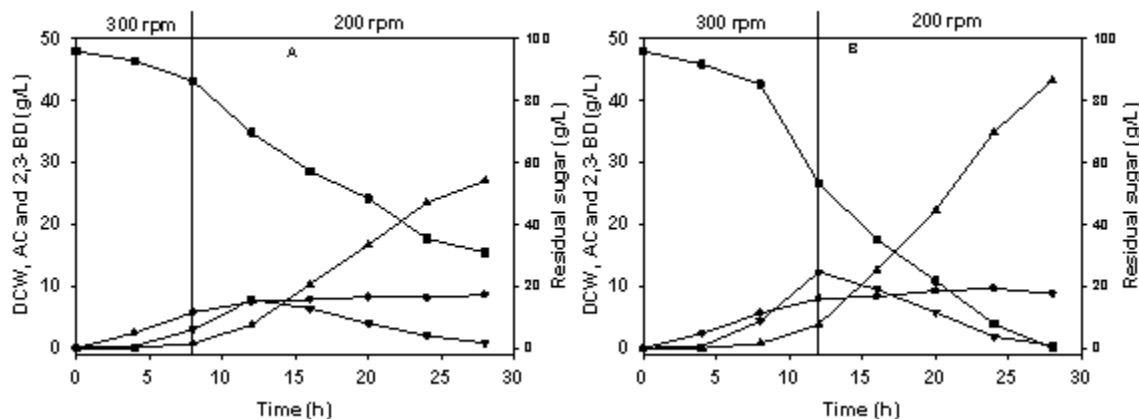
300 rpm, and about 14.80 g/L of AC could be obtained after 28 h. Whereas AC production at 100 and 200 rpm agitation speed exhibited an increasing trend from 4 h to 12 h and then decreased after 12 h (Fig. 4B). The reason might be that excess oxygen at the agitation speed of 300 rpm oxidized NADH *via* the respiratory chain and resulted in the decrease of available NADH, thus limiting the conversion from AC to 2,3-BD by BDH *in vivo* (Yang *et al.* 2013). As reported in previous studies, oxygen supply played an important effect on 2,3-butanediol production (Zeng *et al.* 1994; Zhang *et al.* 2010a; Yang *et al.* 2013). Under anaerobic conditions, excess reducing equivalents produced during the fermentation process resulted in more carbon flux into byproduct such as ethanol and lactate. Under conditions of adequate oxygen supply, the TCA cycle would be active and compete for the intermediate pyruvate with the 2,3-BD pathway, leading to decreased 2,3-BD production (Zeng *et al.* 1994; Zhang *et al.* 2010a). Actually, microbial 2,3-BD production belongs to microaerobic fermentation. To increase 2,3-BD yield, Zeng *et al.* (1994) employed respiratory quotient as a control parameter for optimum oxygen supply and scale-up of 2,3-BD production under microaerobic conditions, and 2,3-BD yield was significantly increased by the strategy. During the batch fermentation process, the sugar consumption rate was relatively higher at 300 rpm and the sugar in the broth was completely depleted after 28 h. The residual sugar of 21.85 g/L and 32.65 g/L remained in the broth at 200 and 100 rpm, respectively after 28 h (Fig. 4D).

The above results of batch fermentation using different agitation speeds indicated that the higher biomass concentration, sugar consumption rate, and carbon flux into 2,3-BD pathway (AC and 2,3-BD) could be obtained at the agitation speed of 300 rpm, but excess oxygen supply led to AC accumulation partially due to low available NADH limiting the reaction from AC to 2,3-BD. In contrary, the agitation speed at 200 rpm could decrease the oxygen supply and efficiently promote the conversion of AC to 2,3-BD. Therefore, the agitation speed was first controlled at 300 rpm to achieve higher biomass and carbon flux into 2,3-BD pathway, and subsequently it was switched to 200 rpm to reduce the AC accumulation. Higher 2,3-BD yield might be achieved in comparison to single agitation speed control conditions as described previously (Ji *et al.* 2009).

### Two-Stage Agitation Speed Control Strategy for 2,3-BD Production

A two-stage agitation speed control strategy was proposed according to the data analysis described above. Figure 4B showed that rapid accumulation of AC was observed during 8 h to 12 h period. Hence, the agitation speed was set at 300 rpm in the first 8 h or 12 h and then switched to 200 rpm until the end of fermentation. The results are presented in Fig. 5.

When the agitation speed was controlled at 300 rpm in the first 8 h, and then changed to 200 rpm, a 2,3-BD concentration of 27.06 g/L was obtained and similar to the single-agitation speed of 300 rpm but the AC concentration was kept at low level (0.79 g/L) (Fig. 5A). However, when the agitation speed was controlled at 300 rpm in the first 12 h and then switched to 200 rpm, a maximum 2,3-BD concentration of up to 43.32 g/L with the increase of 60% compared with that at 300 rpm, whereas the final AC concentration was only 0.46 g/L at 28 h (Fig. 5B). These results indicated that a two-stage agitation speed control strategy could efficiently improve 2,3-BD production and reduce the byproduct AC accumulation.

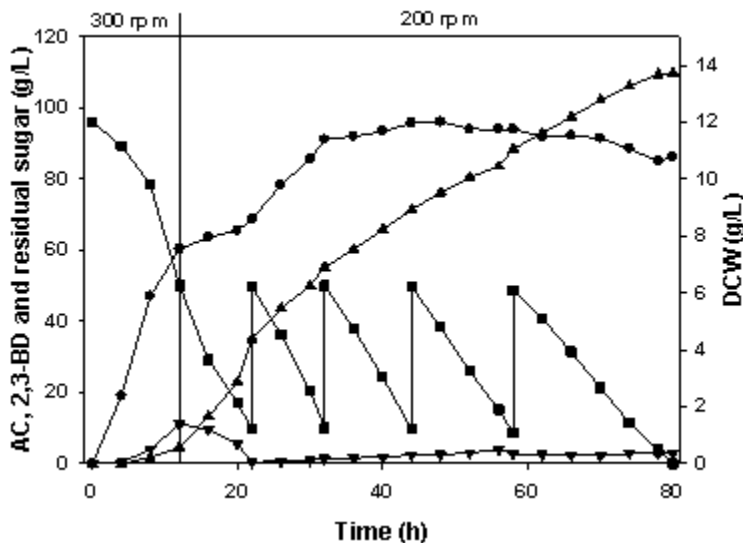


**Fig. 5.** Time-course of DCW (circle), AC (inverted triangle), 2,3-BD (upright triangle), and sugar consumption (square) evolution in batch fermentation by *S. marcescens* H30 using two-stage agitation speed control strategy: (A) 300 rpm for the first 8 h then switched to 200 rpm; (B) 300 rpm for the first 12 h then switched to 200 rpm

### Fed-Batch Fermentation

During the fermentation process, 2,3-BD production is mainly dependent on the conversion of the substrate (Koutinas *et al.* 2016). So fed-batch was required for 2,3-BD production with high yield after the sugar depletion. The fed-batch experiment was carried out in a 5-L bioreactor with 3 L of the optimized SSJ medium using an interim feeding strategy.

The agitation speed was first controlled at 300 rpm and then switched to 200 rpm after 12 h. The raw SSJ was concentrated to 800 g/L of sugar (sucrose, glucose, and fructose) as stock sugar solution and added into the bioreactor when the residual sugar in the broth decreased to 10 g/L. Figure 6 illustrates the changes of four variables including DCW, AC, 2,3-BD, and residual sugar.



**Fig. 6.** Time-course of DCW (circle), AC (inverted triangle), 2,3-BD (upright triangle), and sugar consumption (square) evolution in fed-batch fermentation by *S. marcescens* H30 using two-stage agitation speed control strategy

The results indicated that the cell grew rapidly in the first 12 h and then appeared to grow slow due to the agitation speed changed to 200 rpm. Subsequently the cell density continued to increase and entered the stationary phase at 32 h. The maximum DCW of 11.97 g/L was achieved after 44 h. The main byproduct AC showed a sharp increase before 12 h at the agitation speed of 300 rpm and then decreased obviously when the agitation speed was switched to 200 rpm. The sugar consumption rate and 2,3-BD production in the fed-batch fermentation before 22 h were similar to those in the batch fermentation using the two-stage agitation speed control strategy. Thereafter, the concentration of 2,3-BD continued to increase by feeding additional concentration of SSJ solution. Ultimately, the total sugar of 252.16 g/L was consumed at 78 h. The maximum 2,3-BD concentration of 109.44 g/L with the productivity of 1.40 g/(L·h) and the yield of 83.02 % was achieved after 78 h.

## CONCLUSIONS

1. *Serratia marcescens* H30, an industrial potential microbial strain, was used successfully for the production of 2,3-butanediol.
2. The strain could use sweet sorghum juice as the sole medium to produce 2,3-butanediol.
3. Several exogenous nutrients including nitrogen source and inorganic salts could obviously improve the production of 2,3-butanediol from sweet sorghum juice.
4. An efficient fermentative strategy using fed-batch combined with agitation speed control was developed for 2,3-butanediol, and higher 2,3-butanediol titer was achieved.
5. The sweet sorghum juice was a promising renewable resource for large-scale 2,3-BD production by *Serratia marcescens* H30.

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