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High Cell Density Fermentation of *Saccharomyces cerevisiae* JUL3 in Fed-batch Culture for the Production of β-Glucan

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Abstract: β-Glucan is a cell wall component that is one of the most plentiful cell polysaccharides. Moreover, it has been found to have several beneficial effects on the immune system. In yeast, β-glucan is mainly contained in the yeast cell wall, and thus it is important to produce high levels of cell mass for the mass production of yeast β-glucan. Response surface methodology (RSM) offers a potential means of optimizing process factors and medium components; it has been used to estimate the effects of medium components on cell mass production. In the present study, the optimal concentrations of molasses and corn steep liquor (CSL) in the medium were determined to be 6.4 % (v/v) and 17 % (v/v). The cell mass predicted by statistical analysis was 9.76 g/L after 20 h of cultivation. In a 2.5-L stirred tank reactor (STR), the cell mass produced in a batch culture was 36.5～ 39.3 g/L. The maximum cell mass in the fed-batch cultures of *Saccharomyces cerevisiae* JUL3 was 95.7 g/L using 50 % molasses solution and a feed rate of 10 mL/h. The cell mass obtained in the fed-batch culture was 2.4-fold higher than that obtained in the batch culture.

Keywords: *high-cell-density fermentation, Saccharomyces cerevisiae JUL3, molasses, response surface methodology, fed-batch culture*

Introduction

β-Glucan is a polymeric compound of glucose bonded via β-(1,3)- or β-(1,6)-D-glycosidic linkages [1,2]. It is the most plentiful polysaccharide in the cell wall and can be obtained from bacteria, yeast, fungi, and cereal plants [3]. β-Glucan has several chemotherapeutic effects, which include the inhibition of tumor development, enhancement of defense against bacterial, viral, fungal, and parasitic challenges, and the activation of macrophages [4-7]. Moreover, several studies have been conducted on the protective effect mechanism of β-glucan; one concluded that it is related to the antioxidant capacity of the molecule [8-10]. Because of its pharmacological effects, β-glucan is viewed as a powerful immune response regulator [11].

Yeast β-glucan is contained mainly in the yeast cell wall [1,2]. Therefore, it is important to produce a high cell mass during the mass production of yeast β-glucan, which is achieved by optimizing the production media. Statistical analysis offers tools for optimizing medium components; the response surface methodology (RSM) is probably the most extensively used statistical method for optimizing medium components. RSM can be used to determine optimal conditions, ranges of controllable variables, and to generate polynomial equations. It can be also used to estimate relationships between controllable variables and observed results [12,13].

Fed-batch cultures have many advantages, such as a high cell concentration; they are used widely in the bio-industry. Fed-batch cultures may be divided into four fed-batch types: intermittent, constant, exponential, and optimized types [14-16]. In fed-batch cultures, cell mass and productivity are maximized by controlling culture conditions such as the temperature and pH, the composi-

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tion of the feed media, and the substrate feed rate.

In a previous study, *Saccharomyces cerevisiae* JUL3, which produces a high content of β-glucan, was developed by UV mutagenesis and laminarinase resistance [17]. In the present study, the production medium was optimized by RSM using molasses and corn steep liquor in a shake-flask culture to increase the cell mass of *S. cerevisiae* JUL3. In a 2.5-L stirred tank reactor (STR), the culture conditions, such as the agitation speed and aeration rate, were tested and fed-batch cultures were performed using various concentrations of molasses and different feeding rates.

Experimental

Microorganism

The microorganism used in this study was *Saccharomyces cerevisiae* JUL3, which was isolated from *S. cerevisiae* JH (Hansen 1883) by UV mutagenesis [17] and subcultured with YPD agar at 30 ^{o}C.

Medium and Cultivations

The seed medium was composed of 2.0 % (w/v) glucose, 1.0 % (w/v) yeast extract, and 2.0 % (w/v) Bacto-peptone. Seed culture was carried out at 30 ^{o}C for 16 h in a shaking incubator at 200 rpm. The composition of the basal medium was as follows: 3.0 % (w/v) glucose, 1.0 % (w/v) yeast extract, 2.0 % (w/v) Bacto-peptone, 0.1 % (w/v) $MgSO_4 \cdot 7H_2O$, and 0.5 % (w/v) KH_2PO_4 (pH 6.0). Basal medium was substituted by molasses [5 % (w/v) glucose, 5 % (w/v) fructose, and 40 % (w/v) sucrose] and corn steep liquor (CSL) as carbon and nitrogen sources and optimized by using RSM. The main cultures were inoculated with 2.0 % seed culture. In a 2.5-L stirred tank reactor (KF-10 L, Kobiotech Co. Ltd., Korea), the optimized concentrations of molasses and CSL were 6.4 % (v/v) and CSL 17 % (v/v), respectively. The operating conditions used were 30 ^{o}C, 1.0 vvm, and 200 rpm. In the fed-batch cultures, the working volume was 1.2 L and the final volume reached 2 L. Various concentrations of molasses solutions (30, 50, and 75 %) were fed continuously at rates of 10 or 20 mL/h using a peristaltic pump (MP-3N, Tokyo Rikakikai Co. Ltd., Japan) after 18 h of cultivation.

Experimental Design for Shake-Flask Cultures

Response surface methodology was used to determine the optimal concentrations of molasses and CSL in the shake-flask cultures. The variables were assigned according to equation (1).

$$x_i = (X_i - X_0)/\Delta X_i \quad i = 1, 2, 3..., j \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value.

The behavior of the system was explained by the following second-order polynomial equation (2):

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \tag{2}$$

where y is the predicted result, and β_0 (offset term), β_i (linear effect), β_{ii} (squared effect), and β_{ij} (interaction effect) are constant coefficients; x represents the coded level of the independent variable. The SAS 9.1 package was used for regression analysis of the experimental data and to estimate the regression equation coefficients.

Analytical Methods

Glucose levels were measured using high-performance liquid chromatography (HPLC, YOUNG-LIN Instrument Co. Ltd., Korea) with a ZORBAX NH_2 column (21.2 × 250 mm, Agilent Technologies, Inc., USA) and a refractive index detector (YOUNG-LIN Instrument Co. Ltd., Korea). The column temperature was maintained constant at 50 ^{o}C. Acetonitrile (80 %) and water (20 %) were used as the mobile phase at a flow rate of 1.0 mL/min. The dry cell weight (DCW) was calculated using the relationship between the absorbance at 660 nm and DCW. Total glucan, α-glucan, and β-glucan were analyzed using a Yeast β-Glucan kit (K-YBGL, Megazyme International Ireland Ltd., Ireland) according to a reported assay procedure [18].

Results and Discussion

Optimization of Carbon and Nitrogen Sources by Statistical Analysis

The organic nitrogen sources of basal medium are inadequate for industrial fermentation because of their high cost. When glucose was fed as a sole carbon source at a concentration lower than 1.0 % in the fed-batch culture, the cell mass concentration during the fed-batch culture did not increase. Several researchers have reported this phenomenon [19-21]. Molasses is a byproduct of sugar production and CSL contains vitamins and minerals. They have been used successfully for fermentations. Moreover, many papers have reported that molasses and CSL are economical sources for fermentation process [22-24]. Kim and coworkers (2002) reported that CSL is a good source for the production of acetic acid by *Clostridium thermoaceticum* [25]. Therefore, molasses and CSL were used herein as carbon and nitrogen sources.

Response surface methodology (RSM) was used to de-

Table 1. Range of Variables at Different Levels for Experimental Design and Results

Factor	Symbol	Coded values				
		-1.414	-1	0	+1	+1.414
Molasses	X_1 (%,v/v)	3.17	4	6	8	8.83
C.S.L	X_2 (%, v/v)	7.93	10	15	20	22.1

Runs	Coded values		
	X_1	X_2	Dry cell weight (g/L)
1	+1	+1	9.47
2	+1	-1	8.50
3	-1	+1	9.01
4	-1	-1	7.29
5	+1.414	0	9.12
6	-1.414	0	8.05
7	0	+1.414	9.38
8	0	-1.414	7.87
9	0	0	8.21
10	0	0	9.92
11	0	0	9.11
12	0	0	8.25

termine the optimal concentrations of molasses and CSL. The experiment was performed using two independent variables - molasses (X_1) and CSL (X_2) - in a 2^2 full factorial design experiment with four star points ($\alpha = \pm 1.414$) and four replicates at the center point. The experimental design and results after 20 h of cultivation are presented in Table 1.

Regression analysis was performed to fit the response function to the experimental data (Table 2). The coefficient of determination ($R^2 = 0.938$) was high, indicating that the response equation provided a suitable model for the response surface of cell mass production, and the coefficient of variation was low (3.5 %), indicating the high degree of accuracy and the high reliability of the experiment [12,13]. The response equation obtained was

$$y = 9.514 + 0.399x_1 + 0.604x_2 - 0.473x_1^2 - 0.455x_2^2 - 0.191x_1x_2$$

where x_1 = coded value of molasses, x_2 = coded value of CSL, and y = dry cell weight.

Figure 1 shows a contour plot of the calculated response surface. The optimal points for variables were determined for maximum dry cell weight. The optimum values for molasses (x_1) and CSL (x_2) for the production of cell mass were 0.213 and 0.425, respectively. The optimal molasses and CSL concentrations for the production of cell mass were 6.4 and 17.0 %, respectively. The maximum value of cell mass predicted using the model was 9.76 g/L after 20 h of cultivation.

Figure 2 compares the cell growths of basal and optimal

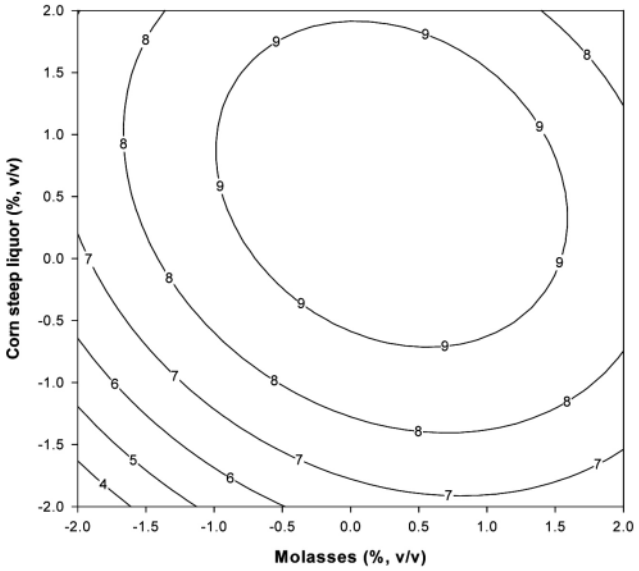

Figure 1. Contour plot of the calculated response surface on cell mass production.

Table 2. Statistical Analysis for the Model of Cell Mass Production at Different Concentrations of Molasses and CSL

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P* > *F*
Model	5.714	5	1.143	12.04	0.016
Error	0.379	4	0.095		
Corrected total	6.093	9			

Coefficient of variation (CV) = 3.512 %; coefficient of determination (R^2) = 0.938

Model term	Parameter estimate	Degree of freedom	Computed t	P (P > | t |)
Intercept	9.514		43.68	< .0001
X_1	0.399	1	3.66	0.0215
X_2	0.604	1	5.54	0.0052
X_1*X_2	-0.191	1	-3.28	0.0304
X_{11}	-0.473	1	-3.16	0.0343
X_{22}	-0.455	1	-1.24	0.2826

media. When retesting was performed to confirm the optimal conditions, a cell mass of 10.8 g/L was obtained after 20 h of cultivation. Moreover, the production of cell mass in the optimal medium was higher than in basal medium containing 3.0 % glucose, and the maximum cell mass obtained was 18.8 g/L after 32 h of cultivation. The difference in the growth rates of *S. cerevisiae* JUL3 was due to the presence of sucrose, which is the major carbon source in molasses. Sucrose degraded to glucose and fructose, which were utilized as carbon sources. Therefore, despite the late cell growth, a higher cell mass was obtained at a lower glucose concentration [21].

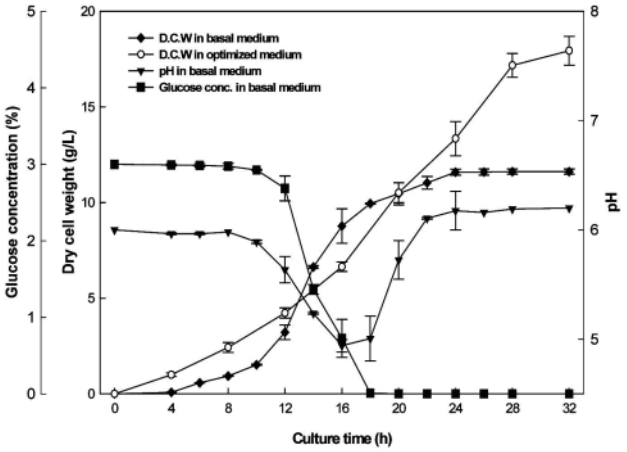

Figure 2. Comparison of *S. cerevisiae* JUL3 cell growth using basal or optimized media in a shake-flask culture. All cultures were conducted at 30 °C, 200 rpm, and pH 6.0.

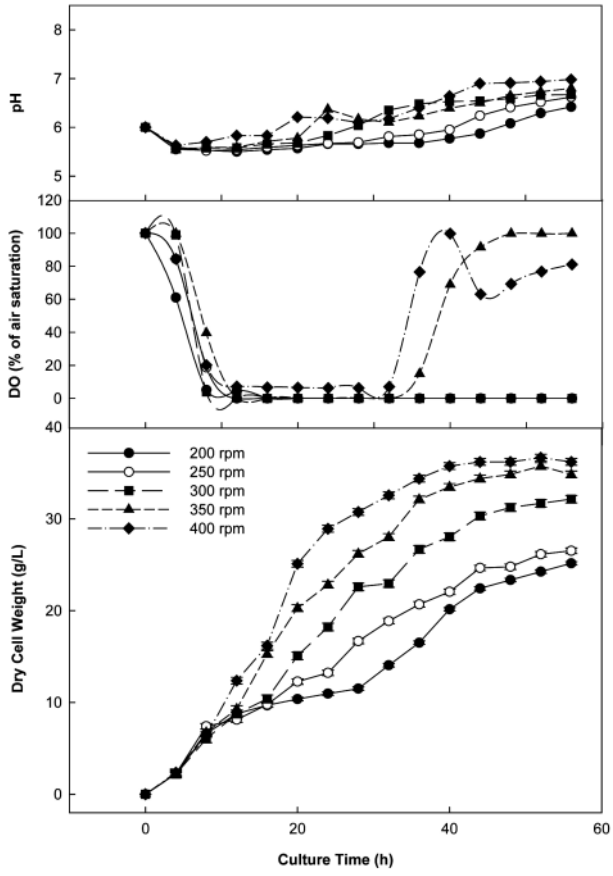

Figure 3. Effect of agitation speed on cell mass production in a 2.5-L STR. Optimized medium containing molasses and CSL was used. All cultures were conducted at 30 °C, 1.5 vvm, and pH 6.0.

Production of β-glucan

The compositions of the cell walls changed according to medium components and the culture conditions, [26,27]. The content of β-glucan in the yeast cells produced was therefore analyzed using a Megazyme β-Glucan kit. As shown in Table 3, the β-glucan contents of *S. cerevisiae* JUL3 produced using basal or optimal media were 97.2 and 92.9 mg/g of dry cell weight, respectively. Thus, the effect of the carbon and nitrogen sources on the β-glucan composition of the yeast cell wall was slight.

Table 3. Comparison of the Glucan Contents of *S. Cerevisiae* JUL3 Produced Using Basal and Optimized Media

Type of medium	Content of glucan (mg/g of dry cell weight)		
	α-Glucan	β-Glucan	Total glucan
Basal medium	3.5 ± 0.25	97.2 ± 0.053	100.7 ± 0.044
Optimized medium	5.2 ± 0.17	92.9 ± 0.01	98.1 ± 0.017

Batch Culture in a 2.5-L Stirred Tank Reactor (STR)

For the 2.5-L STR, the culture conditions, such as the agitation speed and aeration rate, are essential factors for batch culture [28]. The effects of the agitation speed (200 ~ 400 rpm) and aeration rate (1.0 ~ 3.0 vvm) on the production of cell mass were investigated. The temperature and pH were maintained at 30 °C and pH 6.0, respectively. As the agitation speed increased, the cell mass increased (Figure 3). Dissolved oxygen (DO) decreased rapidly to almost zero during the early fermentation stage (8 ~ 12 h) and remained low until the end of the fermentation, except at 350 and 400 rpm where the DO levels increased after 32 h of cultivation, presumably due to carbon source exhaustion. The maximum cell masses at 350 and 400 rpm were similar (35.1 and 36.9 g/L, respectively), showing that 350 rpm may be a more suitable rate for the fed-batch culture. Increased aeration elevated the oxygen uptake rate (OUR) and the oxygen transfer rate (OTR), showing that a high cell mass is affected by OUR and OTR [19]. Figure 4 shows the effect of the aeration rate on the cell mass production. The effect of the aeration rate on the production of cell mass was slight; cell masses of 34.6 ~ 39.3 g/L were obtained.

Fed-batch Culture

Kim and Yun (2006) investigated the use of a Fed-batch culture for β-glucan production using a concentrated feeding medium [29]. Calado and coworkers (2003) reported that high glucose concentrations induce alcoholic fermentation in yeast cell cultures [21], which is unfavorable for the growth of *S. cerevisiae*. Molasses (containing mainly sucrose) was therefore used in our fed-batch cultures to increase cell mass. The temperature and pH were maintained at 30 °C and pH 6.0, respectively. The concentrations of molasses in the feeding media were 30, 50, and 75 %. The continuous fed-batch method was used as a feeding strategy for

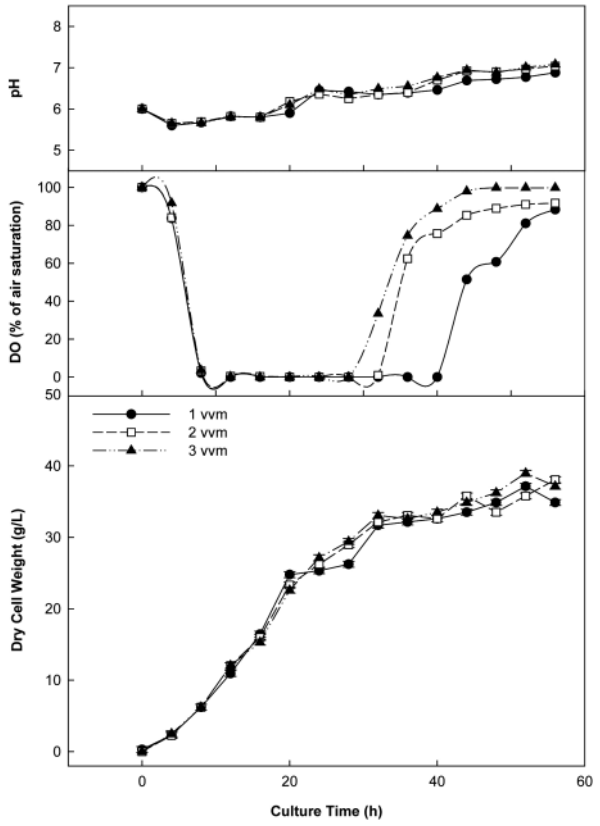

Figure 4. Effect of aeration rate on cell mass production in a 2.5-L STR. Optimized medium containing molasses and CSL was used. All cultures were conducted at 30 °C, 350 rpm, and pH 6.0.

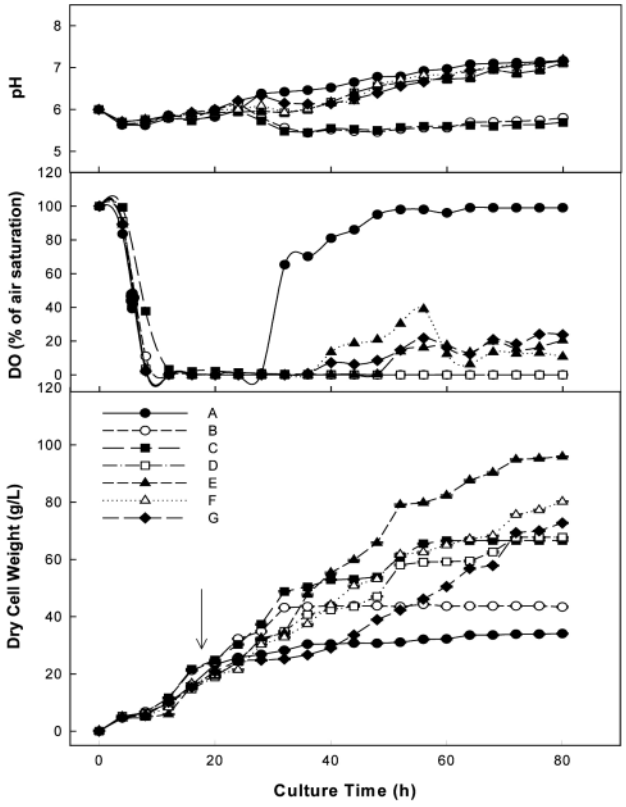

Figure 5. Fed-batch cultures in a 2.5-L STR. All cultures were conducted at 30 °C, 350 rpm, 1.5 vvm, and pH 6.0. The arrow indicates the point at which feeding began. A: Batch culture; B: 75 % molasses and a feeding rate of 20 mL/h; C: 75 % molasses and a feeding rate of 10 mL/h; D: 50 % molasses and a feeding rate of 20 mL/h; E: 50 % molasses and a feeding rate of 10 mL/h; F: 30 % molasses and a feeding rate of 20 mL/h; G: 30 % molasses and a feeding rate of 10 mL/h.

molasses at feeding rates of 10 and 20 mL/h. The results obtained from fed-batch cultures of *S. cerevisiae* JUL3 are shown in Figure 5. DO (%) decreased rapidly to zero at 8～12 h, as it did in the batch culture. DO increased rapidly after 28 h of cultivation in the batch culture, but it was maintained below ca. 20 % during the fed-batch culture. In terms of the different feeding strategies, the maximum cell mass (95.7 g/L) and productivity (1.32 g/L・h) were obtained at 50 % molasses and a feeding rate of 10 mL/h. When 75 % molasses was fed, the production of cell mass did not increased. At 30 % molasses, the feeding rate was 20 mL/h, and at 75 % the feeding rate was 10 mL/h, corresponding to cell masses of 80.4 and 66.8 g/L, respectively.

Conclusions

In this study, molasses and corn steep liquor (CSL) were found to be suitable medium components for the production of cell mass by *S. cerevisiae* JUL3. The response surface methodology (RSM) was used to optimize the carbon and nitrogen sources to maximize cell mass production. The optimal values obtained by statistical analysis were 6.4 % (v/v) molasses (x_1 = 0.213) and 17 % (v/v) CSL (x_2 = 0.425), which gave a predicted cell mass of 9.76 g/L. When the effects of the agitation speed and aeration rate in a 2.5-L STR were investigated, the cell mass was found to be affected mainly by the agitation speed. The cell mass of *S. cerevisiae* JUL3 obtained from a fed-batch culture (95.7 g/L) was much higher than that obtained from a batch culture.

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