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Optimization of medium for pullulan production using a novel strain of *Auerobasidium pullulans* isolated from sea mud through response surface methodology

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ABSTRACT

In this study, response surface methodology (RSM) was used to optimize the medium based on the Plackett–Burman and Central-Composite Designs for the production of pullulan using a novel strain of *Auerobasidium pullulans* CJ001 isolated from sea mud from Eastern China for the first time. NaCl, K_2HPO_4, and $(NH_4)_2SO_4$ were found to have significant effects on pullulan production using the Plackett–Burman Design. The concentrations of the three above mentioned compounds were further optimized using the Central-Composite Design. Results showed that the final concentration of medium optimized using RSM was 1.98 g/L NaCl, 0.77 g/L K_2HPO_4, and 1.0 g/L $(NH_4)_2SO_4$. Production of pullulan reached 26.13 g/L under the optimized medium. The structure of pullulan was confirmed by Fourier transform infrared spectroscopy (FTIR) and High Performance Liquid Chromatography (HPLC).

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1. Introduction

Pullulan, an exocellular polysaccharide produced by *Aureobasidium pullulans*, is a linear mixed linkage α-D-glucan primarily consisting of maltotriose repeating units interconnected by α-(1 → 6) linkages. Pullulan is highly valued for its applications as a coating and packaging material, a sizing agent for paper, and a starch replacement in low-calorie food formulations, in cosmetic emulsions, and in other industrial products (Singh, Saini, & Kennedy, 2008). Therefore, the search for better pullulan-producing strains is very important.

A number of sea mud samples were collected from Eastern China, from which 113 strains were isolated in a laboratory. *A. pullulans* CJ001 was found to produce a large amount of extracellular polysaccharide without producing melanin during fermentation. Therefore, the optimization of the composition of the medium used for the pullulan production of this new pullulan-producing strain was conducted in the current study.

2. Materials and methods

2.1. Microorganism

A. pullulans CJ001, isolated from the sea mud in the east of China, was maintained at 4 °C on potato dextrose agar (PDA) and subcultured every 2 weeks.

2.2. Medium preparation

The medium contained 50 g sucrose, 2.0 g yeast extract, 0.77 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1.98 g NaCl in 1 L distilled water. The pH was adjusted to 5.5, and the medium was autoclaved at 121 °C for 15 min.

2.3. Fermentation

Seed cultures were prepared by inoculating cells grown on a PDA slant into 250 mL flask containing 50 mL of inoculum medium and were subsequently incubated at 28 °C for 48 h while being shaken at 200 rpm. Then, 2.5 mL of seed culture was placed in a 250 mL flask containing 50 mL of fermentation media. The seed culture was shaken at 200 rpm and at 28 °C for 96 h (Wu, Jin, Tong, & Chen, 2009).

2.4. Isolation and purification of pullulan

The culture was centrifuged at 15,000 × *g* for 20 min to remove the microorganisms. An aliquot (3 mL) of the supernatant was transferred into a test tube, and then mixed thoroughly with 6 mL cold ethanol. The prepared mixture was left in a refrigerator (4 °C) for 12 h to precipitate the exocellular polysaccharide. Residual ethanol was removed carefully, then 3 mL deionized water was added and the mixture was heated to 80 °C in a water bath to dissolve the precipitate. The solution was dialyzed against deionized water for 48 h to remove small molecules. The polysaccharide was reprecipitated by adding 6 mL cold ethanol, and was

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Table 1
The Plackett–Burman Design for screening nutrient in pullulan production.

Variable	Nutrient	Levels		*F*-value	$P > \|F\|$	Ranking
Nutrient code		Low (−1)	High (+1)			
A	NaCl (g/L)	8	12	37.78	0.0017	1**
B	K_2HPO_4 (g/L)	1	3	15.28	0.0113	2*
C	Sucrose (g/L)	0.5	1.5	4.10	0.0988	6
D	$(NH_4)_2SO_4$ (g/L)	0.4	0.8	10.35	0.0236	3*
E	Yeast extract (g/L)	4	6	7.31	0.0426	5*
F	MgSO4·$7H_2O$ (g/L)	0.1	0.3	8.60	0.0325	4*

* Statistically significant at 95% of probability level.
** Statistically significant at 99% of probability level.

recovered by filtering the mixture through pre-weighed Whatman GF/A filter paper. The filter paper with the recovered precipitate was dried at 80 °C to a constant weight (Badr-Eldin, El-Tayeb, El-Masry, Mohamad, & El-Rahman, 1994). The pullulan content of the ethanol precipitate was determined using the coupled-enzyme assay technique described by Israilides, Bocking, Smith, and Scanlon (1994). Pullulan content was expressed as g/L.

2.5. Analytical methods

Pullulan molecular weight (Mw) was determined by High Performance Gel Filtration Chromatography (HPGFC) (LC-10A, Shimadzu, Japan) on an Ultrahydrogel Size Exclusion Column (LKB-Prodokter, AB, Bromma, Switzerland), which is capable to detect Mw in the range of 10^3–10^6. In the size exclusion chromatography studies, 0.1 N $NaNO_3$ was used as an eluent at a flow rate of 0.9 mL/min. The detector used was a High Sensitive Refractive Index Detector, Model ERC-7515 A (ERC Inc., Japan). The calibration of the detector was done with known concentrations of commercially available pullulan (Sigma). An aliquot of 20 μL was injected to the column after filtration through 0.45 μm millipore filter, at ambient temperature and the procedure was repeated three times. The software used was the Multi-channel Chromatography Data Station (Version 144A, 1993–1997 Ampersand Ltd.) (Jiang, Wu, & Kim, 2011). FTIR spectra of representative pullulan samples were collected in KBr pellets on a Nicolet Nexus FTIR 470 spectrophotometer over a wavelength range of 400–4000 cm^{-1}. The representative pullulan solutions (3%, w/w) were hydrolyzed at pH 5.0 and temperature 45 °C for 6 h by 10 ASPU/g pullulanase (Wu, Chen, Tong, Xu, & Jin, 2009). The composition of the sugar in the hydrolysates was analyzed by Water600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mm id × 300 mm) used pure water as mobile phase at a flow rate of 0.5 mL/min and the column temperature was maintained at 85 °C. The second column (SpherisorbNH_2, 4.6 mm id × 250 mm) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 mL/min and the column temperature was 30 °C. The detector sensitivity was 4 and the inject volume was 10 μL (Wu, Jin, et al., 2009).

2.6. Experimental design

To optimize the composition of medium, a two-step design, including the Plackett–Burman Design (PBD) and the Central-Composite Design (CCD), was used. In this study, PBD was used to screen the critical variables of the medium parameters, and CCD was carried out for optimizing the important factor and maximizing the pullulan yield. In the PBD, a set of 12 experiments were carried out. The following six medium parameters were chosen for screening design: NaCl, K_2HPO_4, sucrose, $(NH_4)_2SO_4$, yeast extract, and $MgSO_4 \cdot 7H_2O$. Each factor was varied at two levels, coded as −1 and +1 (Table 1). Once the three critical variables were obtained, a

Table 2
The Plackett–Burman Design variables (in coded levels) with pullulan production as response.

Run	Variable levels						Pullulan (g/L)
	A[a]	B	C	D	E	F	
1	+1	+1	−1	+1	+1	−1	22.13
2	+1	−1	−1	−1	+1	+1	18.92
3	−1	+1	+1	+1	−1	+1	19.65
4	−1	+1	−1	−1	−1	+1	15.63
5	+1	−1	+1	+1	−1	+1	19.92
6	+1	−1	+1	−1	−1	−1	20.27
7	−1	+1	+1	−1	+1	−1	16.05
8	−1	−1	−1	−1	−1	−1	15.48
9	+1	+1	+1	−1	+1	+1	17.26
10	−1	−1	+1	+1	+1	−1	19.81
11	+1	+1	−1	+1	−1	−1	19.42
12	−1	−1	−1	+1	+1	+1	18.02

[a] The symbols were the same as those in Table 1.

Table 3
The Central-Composite Design for optimizing nutrient concentration.

Run	Nutrient						Pullulan (g/L)
	Coded levels			Actual levels			
	X_1	X_2	X_3	X_1	X_2	X_3	
1	0	0	0	2	0.8	1.0	25.37
2	0	0	0	2	0.8	1.0	25.23
3	−1	+1	0	1.6	1.2	1.0	16.47
4	0	−1	−1	2	0.4	0.8	18.25
5	+1	−1	0	2.4	0.4	1.0	19.69
6	0	−1	+1	2	0.4	1.2	19.26
7	+1	0	−1	2.4	0.8	0.8	16.80
8	0	+1	−1	2.4	0.8	1.2	15.59
9	−1	0	−1	1.6	0.8	0.8	16.80
10	0	0	0	2	0.8	1.0	24.89
11	+1	+1	0	2.4	1.2	1.0	18.70
12	0	0	0	2	0.8	1.0	25.69
13	0	+1	−1	2	1.2	0.8	18.82
14	+1	0	+1	1.6	0.8	1.2	18.92
15	0	0	0	2	0.8	1.0	26.81
16	0	+1	+1	2	1.2	1.2	16.23
17	−1	−1	0	1.6	0.4	1.0	17.63

X_1 = NaCl (g/L), X_2 = K_2HPO_4 (g/L), X_3 = $(NH_4)_2SO_4$ (g/L).

CCD was carried out to fit a polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 \times X_2 + \beta_{13} X_1 \times X_3 + \beta_{23} X_2 \times X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \quad (1)$$

where Y is the yield of pullulan, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are interaction coefficients, β_{11}, β_{22} and β_{33} are squared coefficients, and X_1, X_2 and X_3 are coded independent variables. The Design Expert (Version 7.1.6, State-Ease Inc., Minneapolis, USA) software was used for the experimental design, data analysis, model building, and graph plotting.

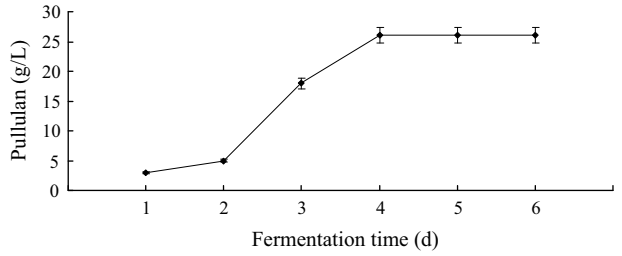

Fig. 1. Effect of fermentation time on pullulan production. Data are shown as mean ± SD ($n = 3$).

3. Results and discussion

3.1. Screening of significant nutrients using a Plackett–Burman Design (PBD)

PBD is a powerful technique for screening critical variables, and therefore, it was used to analyze the effect of six variables on pullulan production in this study (Table 1). In the experiment design, twelve experiments were conducted and the results are shown in Table 2. The analysis of variance (ANOVA) for the experiment design was calculated, and significant levels of each medium variable were determined by *F*-test (Table 1). The *F*-test result indicated that NaCl, K_2HPO_4, and $(NH_4)_2SO_4$ significantly affected pullulan production (Table 1). All the other insignificant variables were disregarded and the optimum combination of these three variables was further analyzed using a Central-Composite Design (CCD).

3.2. CCD and response surface analysis

Based on the results of PBD, the optimum combination of three variables, i.e., NaCl, K_2HPO_4, and $(NH_4)_2SO_4$, which significantly affect pullulan production, were further investigated for their optimum combination using a CCD. The design and results of the experiments conducted using a CCD are shown in Table 3. Results were analyzed using ANOVA, and the regression model was obtained as follows:

$$Y = -236.07925 + 139.24688X_1 + 49.91188X_2 + 209.3750088X_3 - 0.25563X_1 \times X_2 - 13.125003X_1 \times X_3 - 11.2500X_2 \times X_3 - 31.82969{X_1}^2 - 24.79844{X_2}^2 - 87.25625{X_3}^2 \quad (2)$$

where Y is the pullulan yield (g/L), X_1 is the NaCl concentration (%), X_2 is the K_2HPO_4 concentration, and X_3 is the $(NH_4)_2SO_4$ concentration. The statistical significance of Eq. (1) was verified through ANOVA for the response surface quadratic model, and the results are summarized in Table 4. The data in Table 4 indicate that the model was highly significant, as demonstrated by the *F*-value and the probability value [$(P > F) < 0.0001$]. The accuracy of fit was proven by the high multiple correlation coefficient ($R^2 = 98.89\%$), indicating that the response model can explain 98.89% of the total variations. Generally, a regression model having an R^2 value greater than 0.9 is considered to have a very high correlation (Haaland, 1989). The value of the adjusted multiple correlation coefficient ($R^2_{Adj} = 97.45\%$) was also sufficiently high to indicate the significance of the model.

Table 4
Analysis of variance for the experimental results of the Central Composite Design.

Factor*	Sum of square	Degree of freedom	*F* value	*P* > F	Significance
X_1	109.77	1	256.68	<0.0001	**
X_2	0.022	1	0.050	0.8288	
X_3	4.44	1	10.38	0.0146	*
${X_1}^2$	109.20	1	255.35	<0.0001	**
${X_2}^2$	66.29	1	155.00	<0.0001	**
${X_3}^2$	51.29	1	119.93	<0.0001	**
$X_1 \times X_2$	7.225E−003	1	0.017	0.9002	
$X_1 \times X_3$	4.41	1	10.31	0.0148	*
$X_2 \times X_3$	3.24	1	7.58	0.0284	*
Model	265.82	9	69.06	<0.0001	**
Lack of fit	0.83	1	0.51	0.6969	
Pure	Error	2.17	4		

* Statistically significant at 95% of probability level.
** Statistically significant at 99% of probability level.

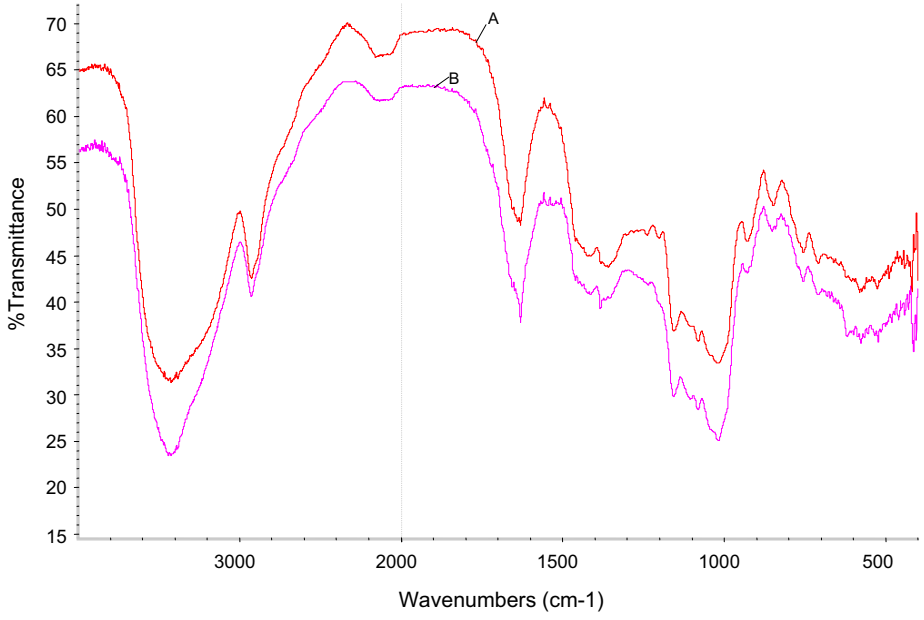

Fig. 2. FTIR spectra of the pullulan prepared in this experiment (A) and obtained from Japan Pharmacopoeia (B).

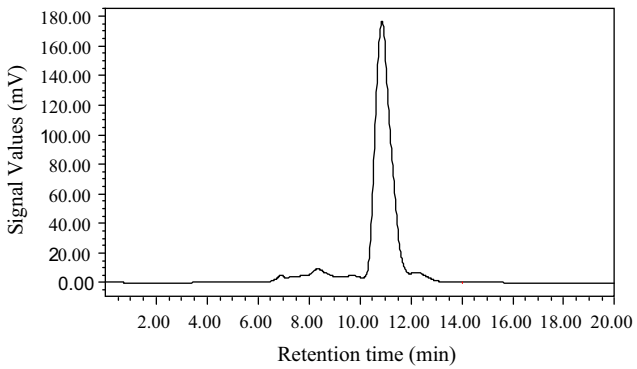

Fig. 3. HPLC profile of the hydrolysates.

The interaction between NaCl and K_4HPO_4 is not significant, while that between NaCl and $(NH_4)_2SO_4$ and that between K_4HPO_4 and $(NH_4)_2SO_4$ are significant (Table 4). The optimal concentrations for the maximum pullulan production based on the model were calculated as 1.98, 0.77, and 1.0 g/L for NaCl, K_4HPO_4, and $(NH_4)_2SO_4$, respectively. By substituting levels of the factors into the regression equation, the maximum predictable response for pullulan production was calculated and was experimentally verified. The maximum pullulan production obtained experimentally using the optimized medium was 26.13 g/L, which was consistent with the predicted value of 25.64 g/L obtained using a response surface methodology regression study.

Pullulan production using *A. pullulans* CJ001 may increase further if the fermentation conditions are optimized in the future. In other reports, maximum pullulan production was 6 g/L (Roukas & Biliaderis, 1995), 12–14 g/L (LeDuy & Boa, 1982), 25.95 g/L (Vijayendra, Bansal, Prasad, & Nand, 2001), 25.1 g/L (Prasongsuk et al., 2007), and 30.28 g/L (Jiang, 2010), respectively. The differences in maximum pullulan production reported in the literature may be attributable to the differences in the types of strain, composition of fermentation medium and seed culture conditions used. NaCl can affect the metabolism of *A. pullulans* CJ001 by regulating the osmotic pressure, ionic strength, and Na^+/K^+-ATPase. The optimum concentration of NaCl observed here for *A. pullulans* CJ001 is higher than that generally used in other studies (Cheng, Demirci, & Catchmark, 2010; Jiang, 2010; Lacroix, LeDuy, Noel, & Choplin, 1985). In addition to affecting the metabolism of *A. pullulans* CJ001 by regulating the osmotic pressure, ionic strength, and Na^+/K^+-ATPase, K_2HPO_4 provides P for *A. pullulans* CJ001. Thus, the optimal concentration of K_2HPO_4 used is also higher than that generally used in other studies (Cheng et al., 2010; Jiang, 2010; Lacroix et al., 1985). $(NH_4)_2SO_4$ can also affect the metabolism of *A. pullulans* CJ001 by providing N for the microorganism, and the optimum concentration used is in agreement with that reported in other studies (Cheng et al., 2010; Jiang, 2010; Lacroix et al., 1985).

3.3. Time course of fermentation

The time courses for pullulan production using *A. pullulans* CJ001 under the optimal conditions obtained from RSM were monitored for 6 d. As shown in Fig. 1, the production of pullulan sharply increased after 2 d of fermentation. The pullulan production reached a maximum after 4 d.

3.4. Characterization of the exopolysaccharide

Based on HPGFC analysis, the Mw of the exopolysaccharide prepared in this study was found to be 2.7×10^5 Da. FTIR spectra of the exopolysaccharide prepared in this experiment and the pullulan obtained from Japan Pharmacopoeia are identical, indicating that this exopolysaccharide is primarily composed of pullulan (Fig. 2). Maltotriose is the primary sugar form of the hydrolysates of the exopolysaccharide with pullulanase, which is capable of cutting off α-(1 → 6) linkages, indicating that maltotriose is the main repeating unit interconnected by α-(1 → 6) linkages, and thus confirming the pullulan structure of this exopolysaccharide (Fig. 3).

4. Conclusions

The optimized medium composition for the pullulan production using *A. pullulans* CJ001 was found to be 1.98 g/L NaCl, 0.77 g/L K_2HPO_4, and 1.0 g/L $(NH_4)_2SO_4$. Under the optimized conditions, the maximum pullulan production of 26.13 g/L was reached during the fermentation culture of *A. pullulans* CJ001. Predicted values obtained using the model equation were consistent with the experimental production results. Therefore, determining the optimum conditions for pullulan production by employing a statistical experimental design is possible.

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