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Optimization of fermentation conditions for pullulan production by *Aureobasidium pullulan* using response surface methodology

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ABSTRACT

In this study, response surface methodology was used for optimization the fermentation conditions for pullulan production by the strain *Aureobasidium pullulans* SK1002 in shaking flask cultures. The production of pullulan was significantly affected by temperature, fermentation time and initial pH. The optimal cultivation conditions stimulating the maximal pullulan production were as follows: temperature, 28 °C; fermentation time, 5 days and initial pH, 5.5. Under these optimized conditions, the predicted maximal pullulan yield was 30.28 g/L. The application of response surface methodology resulted in a significant enhancement in pullulan production. Results of these experiments indicated that response surface methodology was a promising method for optimization of pullulan production.

1. Introduction

Pullulan is a water soluble polysaccharide produced by *Aureobasidium pullulans*. It is a linear mixed linkage α-D-glucan consisting mainly of maltotriose repeating units interconnected by α-(1 → 6) linkages (Saha & Zeikus, 1989). Pullulan can form thin films that are transparent, oil resistant and impermeable to oxygen. Pullulan can be used as coating and packaging material, sizing agent for paper, starch replacer in low-calorie food formulations, cosmetic emulsions and industrial applications (Deshpande, Rale, & Lynch, 1992).

Factors that influence pullulan production include the fungal strain used (Silman, Bryan, & Leathers, 1990), nature of the carbon source (West & Reed-Hamer, 1991) and nitrogen source (Auer & Seviour, 1990) in the culture medium, dissolved oxygen levels (Gibbs & Seviour, 1996) and fermentor configuration (Wecker & Onken, 1991). It is thought that others of the most important factors in pullulan production is the fermentation temperature (McNeil & Kristiansen, 1990), fermentation time (Vijayendra, Bansal, Prasad, & Nand, 2001) and initial pH (Ono, Yasuda, & Ueda, 1977).

Response surface methodology, an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization. This method had been successfully applied in the optimization of medium compositions (Roseiro, 1992), conditions of enzymatic hydrolysis (Ma & Ooraikul, 1986), and fermentation processes (Kalil, Maugeri, & Rodrigues, 2000). It can give information about the interaction between variables, provide information necessary for design and process optimization, and give multiple responses at the same time.

The aim of this work was to apply statistical methods to optimize the fermentation medium conditions for the improvement of pullulan production by *A. pullulans* SK1002. A central composite design (CCD) was used for the fermentation condition optimization reported here.

2. Materials and methods

2.1. Microorganism

Aureobasidium pullulans SK1002 was used. The microorganism was maintained at 4 °C on potato dextrose agar (PDA) and subcultured every 2 weeks.

2.2. Preparation of medium

Both inoculum and fermentation medium contained (g/L), sucrose, 50.0; yeast extract, 2.0; KH_2PO_4, 5.0; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; NaCl, 1.0 and distilled water l L. The medium was autoclaved for 15 min at 121 °C after the pH of the medium was adjusted to 5.5.

2.3. Fermentation

Seed cultures were prepared by inoculating cells grown on a PDA agar slant into a 250-ml flask that contained 50 ml of the inoculum medium and subsequently incubated at 28 °C for 2 days with

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shaking at 200 rpm. About 2.5 milliliters of the seed culture were transferred into the 250-ml flask containing 50 ml of the fermentation media. The culture was shaken at 28 °C and with 200 rpm for 5 days.

2.4. Isolation and purification of pullulan

The culture was centrifuged to remove the microorganism at 15,000*g* for 20 min. Three milliliters of the supernatant were transferred into a test tube, and then 6 ml of cold ethanol was added to the test tube and mixed thoroughly and held at 4 °C for 12 h to precipitate the exocellular polysaccharide. After removal of the residual ethanol, the precipitate was dissolved in 3 ml of deionized water at 80 °C and the solution was dialyzed against deionized water for 48 h to remove small molecules in the solution. The polysaccharide was precipitated again by using 6 ml of the cold ethanol and the precipitate was filtered through a pre-weighted Whatman GF/A filter and dried at 80 °C to a constant weight (Wu, Jin, & Tong, 2009).

2.5. Analytical methods

The pH of the culture medium was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China). The reducing sugars were estimated by the method of Somogyi (Nelson, 1944). The pullulan content of the ethanol precipitate was determined by the modified coupled-enzyme assay technique and was expressed as g/L (Wu et al., 2009).

2.6. Experimental design

To find the optimal cultivation conditions for pullulan production in batch cultures, the key factors affecting the pullulan production must be determined. Based on the results of our preliminary experiments, the major factors were optimized using response surface methodology (RSM) design (Design-Expert 6). Table 1 shows the ranges of variables of temperature, fermentation time and initial pH for RSM.

3. Results and discussion

A total of 15 experiments with combinations of temperature, time and initial pH were conducted. A central composite design with 3 levels for all the 3 factors: temperature (A), time (B) and initial pH (C) were used for this purpose. The range of the variables is given in Table 1. The experimental design and the results obtained from experiments are shown in Table 2. The results of these experiments were fitted with a second order polynomial equation. The values of regression coefficients were calculated, and the fitted equation (in terms of coded values) for predicting pullulan production (X) was as given below regardless of the significance of the coefficients:

$$X = 30.28 - 0.088 \times A - 0.11 \times B - 0.84 \times C - 2.60 \times A^2 - 2.34 \times B^2 - 2.09 \times C^2 + 0.13 \times A \times B - 1.51 \times A \times C + 1.89 \times B \times C \quad (1)$$

Table 1
Values of coded levels used for the experimental design.

Factors	Symbols	Actual levels of coded factors		
		−1	0	1
Temperature	A	26	28	30
Time	B	4	5	6
Initial pH	C	5	5.5	6

Table 2
Central composite design for the experimental design and results.

Run numbers	A Temperature (°C)	B Time (day)	C Initial pH	Pullulan (g/L)
1	28.00	5.00	4.79	26.89
2	28.00	5.00	5.50	29.45
3	28.00	6.41	5.50	25.05
4	28.00	5.00	5.50	29.60
5	28.00	5.00	5.50	30.20
6	30.00	4.00	6.00	19.30
7	28.00	5.00	5.50	31.35
8	30.00	6.00	5.00	24.05
9	26.00	4.00	5.00	25.20
10	28.00	3.59	5.50	25.35
11	30.00	5.00	5.50	24.55
12	26.00	6.00	6.00	26.05
13	28.00	5.00	5.50	31.60
14	25.00	5.00	5.50	24.80
15	28.00	5.00	6.21	24.50

where A, temperature; B, time and C, initial pH.

The statistical significance of the regression model was checked by *F*-test, and the analysis of variance for the response surface quadratic model is shown in Table 3. The model was highly significant, as manifested by the *F*-value and the probability value [$(P > F) = 0.0043$]. The goodness of fit was manifested by the determination coefficient (R^2). In this case, the R^2 value of 96% indicated that the response model can explain 96% of the total variations. In general, a regression model having an R^2 value higher than 0.9 is considered to have a very high correlation (Haaland, 1989). The value of the adjusted determination coefficient ($R^2_{Adj} = 89\%$) was also high enough to indicate the significance of the model.

The optimum of location, obtained by differentiation of the quadratic model, for achieving maximal pullulan production was A = 28 °C, B = 5 days, and C = 5.5. The predicted optimal pullulan production corresponding to these values was 30.28 g/L. To confirm the goodness of the model for predicting maximal pullulan production, Additional experiments in triplicates using these optimized fermentation condition were carried out. These triplicate experiments yielded an average maximum pullulan of 30.26 g/L. The good agreement between the predicted and experimental values confirms the validity of the model and the existence of optimum point.

The 3D response surfaces plots were employed to determine the interaction of the fermentation conditions and the optimum levels that have the most significant effect on pullulan production. The response surfaces plots based on the model are depicted in Fig. 1, Fig. 2 and Fig. 3. It is clear from Fig. 1 that the minimum response of pullulan production (25.19, g/L) occurred when temperature was at its lowest level. Pullulan production increased considerably

Table 3
Analysis of variance for response surface quadratic model obtained from experimental results.

Source	Sum of squares	*df*	Mean squares	*F*-value	Probability > *F*
Model	149.87	9	16.65	14.71	0.0043
A	0.03	1	0.03	0.03	0.8746
B	0.05	1	0.05	0.04	0.8498
C	2.86	1	2.86	2.52	0.1731
A^2	52.20	1	52.20	46.10	0.0011
B^2	42.19	1	42.19	37.27	0.0017
C^2	33.74	1	33.74	29.80	0.0028
AB	0.03	1	0.03	0.03	0.8696
AC	4.54	1	4.54	4.01	0.1017
BC	7.12	1	7.12	6.29	0.0540
Residual	5.66	5	1.13		
Lack of fit	1.74	1	1.74	1.78	0.2529
Pure error	3.92	4	0.98		
Core total	155.53	14	11.11		

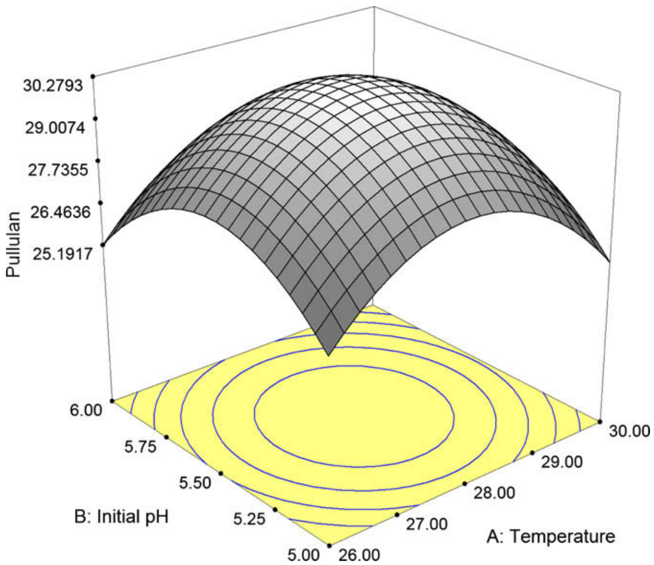

Fig. 1. Response surface for pullulan production by *Aureobasidium pullulans* SK1002. The interaction between initial pH and temperature.

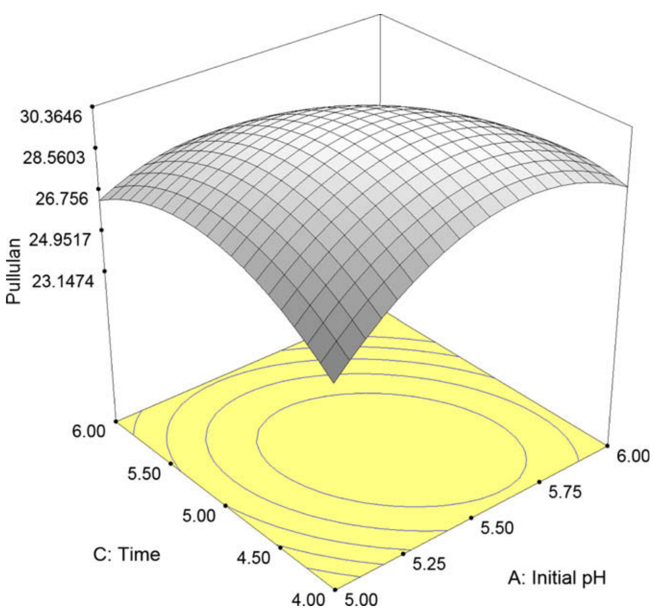

Fig. 2. Response surface for pullulan production by *Aureobasidium pullulans* SK1002. The interaction between initial pH and time.

as temperature increased, indicating that temperature for pullulan production has a significant effect on the responses. As the temperature increased, the responses were maximal nearly at the middle of initial pH. The response was also varied at different levels of initial pH along the axis, suggesting that there is a considerable interaction between temperature and initial pH (Fig. 1). In other reports, optimal conditions for pullulan production were obtained at an initial pH of 5.0 (Vijayendra et al., 2001), 6.0 (Ono et al., 1977), 6.5 (Roukas & Biliaderis, 1995), and 7.5 (Auer & Seviour, 1990). The different optimal initial pH values reported in the literature may be due to the different strains, compositions of fermentation media, and culture conditions used in those studies. Fig. 2 demonstrates the effects of initial pH and time on pullulan production. The pullulan production was affected by the initial pH and time and also there is a considerable interaction between them for pullulan production. Similarly Fig. 3 shows the effects of temperature and

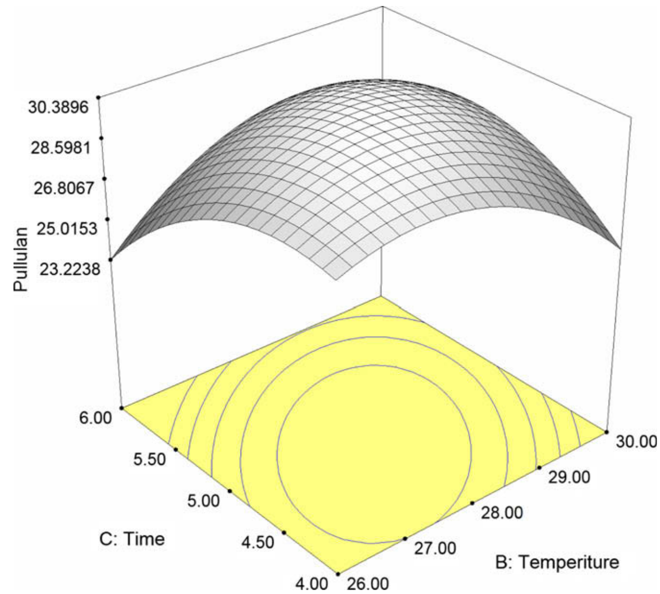

Fig. 3. Response surface for pullulan production by *Aureobasidium pullulans* SK1002. The interaction between temperature and time.

time on the pullulan production. Response surface optimization supported 30.28 g/L production of pullulan by *A. pullulans* SK1002.

4. Conclusions

Statistical optimization of fermentation medium could overcome the limitations of classical empirical methods. It was proved to be a powerful tool for the optimization of the pullulan production by *A. pullulans* SK1002. Response surface methodology was proposed to study the combined effects of culture medium components. The existence of interactions between the independent variables with the responses was observed. The optimum fermentation conditions are as follows: temperature, 28 °C; time, 5 days and initial pH, 5.5. Validation experiments were performed to verify the accuracy of the models, and the results showed that the experimental values agreed with the predicted values well.

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