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Media optimization for elevated molecular weight and mass production of pigment-free pullulan

Xiaoliu Yu, Yulei Wang, Gongyuan Wei*, Yingying Dong

School of Biology and Basic Medical Sciences, Soochow University, Suzhou 215123, China

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ABSTRACT

In this study, an *Aureobasidium pullulans* SZU 1001 mutant, deficient in pigment production, was screened by complex UV and γ-ray mutagenesis. Medium composition optimization for increased pullulan molecular weight and production was conducted using this mutant. Six nutrients: yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $MgSO_4{\cdot}7H_2O$ and $CaCl_2$ were detected within pullulan production in flasks. It is shown that NaCl and K_2HPO_4 have significant influences on molecular weight of pullulan, while yeast extract and $(NH_4)_2SO_4$ significantly affect pullulan yield. To achieve a higher molecular weight and more efficient pullulan production, a response surface methodology approach was introduced to predict an optimal nutrient combination. A molecular weight of 5.74×10^6 and pullulan yield on glucose of 51.30% were obtained under batch pullulan fermentation with the optimized media, which increased molecular weight and pullulan production by 97.25% and 11.04%, respectively compared with the control media.

1. Introduction

Pullulan, is an extracellular water-soluble linear homopolysaccharide composed of maltotriose subunits interconnected via α-(1→6) glycosidic linkages, which endows pullulan with structural flexibility and superior solubility (Leathers, 2003; Lee et al., 1999). It is usually bio-synthesized using strains of the yeast-like polymorphic fungus *Aureobasidium pullulans* (Singh, Saini, & Kennedy, 2008). Owing to its distinctive physical and chemical properties, pullulan has a wide range of commercial and industrial applications in many fields including the food and cosmetic industries, environmental treatment, pharmacy and healthcare, and even lithography (Singh et al., 2008). Furthermore, pullulan also draws considerable interest as a well-defined model substance for basic research (Gupta & Gupta, 2004; Na, Jeong, & Lee, 1997).

Fermentative pullulan production with *A. pullulans* has been widely studied in recent years. Researchers have made many efforts to optimize nutrients such as carbon sources (Barnett, Smith, Scanlon, & Israilides, 1999; Ravella et al., 2010; Wu, Jin, Tong, & Chen, 2009), nitrogen sources (Jiang, Wu, & Kim, 2011; Ravella et al., 2010) and minerals (Gao, Kim, Chung, Li, & Lee, 2010), as well as environmental conditions including pH, temperature, dissolved oxygen, and fermentation methods to increase pullulan production (Chi & Zhao, 2003; Jiang, 2010; West, 2010). During batch or fed-batch pullulan production, the yield of pullulan on substrate (usually glucose or sucrose) is an important indicator of efficient pullulan production, but few studies have focused on these aspects to date. In addition, the molecular weight of pullulan has a significant influence on its utilization. For example, a specific molecular weight of pullulan is required for pharmaceutical applications (Cheng, Demirci, & Catchmark, 2011). Israilides, Scanlon, Smith, Harding, and Jumel (1994) have shown that that the type of agro-industrial wastes used for production had significant influence on the molecular weight of pullulan. Lee et al. (1999) and Wiley et al. (1993) found that phosphates and nitrogen sources affect the molecular weight of pullulan. However, further research on increasing the molecular weight of pullulan through medium optimization (Lin, Zhang, & Thibault, 2007) or using alternative materials such as potato starch waste (Gösungur, Uzunoğullarí, & Dağbağlí, 2011) remains necessary for extending the range of pullulan utilization.

Response surface analysis and orthogonal experimental design are useful and commonly used approaches in fermentative process optimization (Gösungur et al., 2011). Based on the single-factor test, the most favorable combination of factors affecting fermentation can be found and achieved using both approaches through statistical analysis of the data (Chen, Wei, Zhang, & Dong, 2011). In the present study, we began by screening a pigment-free pullulan-producing strain using complex UV and γ-ray mutagenesis. Secondly, detailed studies on media composition to increase pullulan molecular weight and yield were carried out using orthogonal experimental design and response surface

* Corresponding author at: School of Biology and Basic Medical Sciences, Soochow University, Suzhou Industrial Park, Suzhou 215123, China. Tel.: +86 512 65880421; fax: +86 512 65880103.
E-mail address: weigy@suda.edu.cn (G. Wei).

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analysis, respectively. This study is the first detailed work on both aspects of pullulan molecular weight and production using statistical methods, which also presents a potential approach to optimize the production process for related materials.

2. Materials and methods

2.1. Microorganisms

A. pullulans SZU 1001, a strain deficient in pigment production, is a mutant of the melanin-producing strain, *A. pullulans* ATCC 201253. The mutant was screened from a commonly used medium (Seo et al., 2004) after treatment of the parent strain by complex UV and γ-ray mutagenesis (Shao et al., 2010). The strains were maintained in seed medium with 20% (v/v) glycerol at −70 °C.

2.2. Media

The seed medium contained 20% (w/v) potato juice and 20 g/l glucose, with a natural pH. Unless stated otherwise, the initial fermentation medium consisted of 50 g/l glucose, 2.5 g/l yeast extract, 5.0 g/l K_2HPO_4, 0.6 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4{\cdot}7H_2O$, and 1.0 g/l NaCl at pH 6.8 (Seo et al., 2004).

2.3. Complex mutagenesis

Complex mutagenesis of *A. pullulans* ATCC 201253 by UV and γ-ray exposure was performed according to a method described previously (Shao et al., 2010). A 10 ml aliquot of freshly cultured *A. pullulans* ATCC 201253 cells was diluted in 0.9% (w/v) NaCl to a final concentration of 1×10^6 cells/ml. After UV irradiation for 60 s, cells with a survival rate of about 25% were plated on a commonly used medium (Seo et al., 2004) and incubated at 30 °C for 2 days. Colonies without pigment were selected for further cultivation, and the strain with the highest pullulan production was selected for γ-ray mutagenesis, which was carried out by irradiating the strain with γ-ray (Co-60) under an irradiation dosage rate of 80 Gy/min for 7 min. The surviving cells were also plated on the commonly used medium. After complex UV and γ-ray mutagenesis of *A. pullulans* ATCC 201253, a mutant of *A. pullulans* SZU 1001 was screened for further study.

2.4. Culture conditions

Seed cultures were prepared by inoculating cells stored at −70 °C in a 500 ml Erlenmeyer flask containing 50 ml seed medium, and incubated at 30 °C for 24 h on a rotary shaker at 200 rpm. The seeds were then transferred to 500 ml flasks containing 50 ml of fermentation media with an inoculum size of 10% (v/v). Flask fermentation was carried out at 30 °C and 200 rpm on the shaker for 48 h or 72 h, according to the experiment design. Batch fermentation for pullulan production was conducted in a 5 l stirred fermentor (Minifors, INFORS HT, Basel, Switzerland) containing 3 l of fermentation media. The bioreactor was operated at 30 °C and 350 rpm with an aeration rate of 1.0 vvm, and the pH was automatically controlled at 6.8 by adding 3 M H_2SO_4 or 3 M NaOH.

2.5. Analytical methods

Fermentation broth (25 ml) was heated at 80 °C for 15 min in a water bath, and then centrifuged at 8000 × *g* for 20 min to remove cells after being cooled to room temperature. The biomass was determined by drying the cells at 70 °C to a constant weight. Supernatant polysaccharides were precipitated with 2 volumes of ethanol at 4 °C for 12 h. The precipitate was centrifuged at 8000 × *g* and 4 °C for 20 min followed by drying at 80 °C overnight and then weighed (Cheng, Demirci, Catchmark, & Puri, 2011; Chi & Zhao, 2003). Residual sugar concentrations in the supernatant were determined using the dinitrosalicylic acid (DNS) method (Miller, 1959).

The limiting viscosity of pullulan aqueous solutions (in deionized water) was determined using an Ubbelohde capillary viscometer at 25 °C. The intrinsic viscosity [η] was calculated from the Huggins equation. The weight average molecular weight (Mw) of pullulan was calculated from the equation $[\eta] = (0.000258) \times Mw^{0.646}$ (Roukas & Biliaderis, 1995).

Table 1
Difference on pullulan production between the parent strain and the mutant.

Strain	Pigment	Pullulan (g/l)	Mw ($\times 10^6$)
A. pullulans ATCC 201253	Dark (+)	18.03 ± 0.39	1.76 ± 0.13
A. pullulans SZU 1001	Light (−)	18.93 ± 0.71	1.83 ± 0.16

2.6. Experiment design and software

A previously described, two-level, fractional factorial design (FFD) (Almeida e Silva, Lima, Taqueda, & Guaragna, 1998) was introduced to select significant factors within six nutrients that influenced pullulan production. Taguchi orthogonal array design (OAD) is useful for obtaining an optimal medium for pullulan production (Gao et al., 2010). Here, we optimized medium compositions for high molecular weight of pullulan using an $L_9(3^4)$ OAD method. To maximize pullulan yield, response surface methodology (RSM) with a central composite design (CCD) was used owing to its powerful medium optimization function (Gösungur et al., 2011; Khuri & Mukhopadhyay, 2010).

Design Expert software (Version 7.1.6, State-Ease Inc., Minneapolis, MN, USA) and Minitab 16 Statistical Software (Version 16, Minitab, Inc., State College, PA, USA) were used for the experimental design and coefficient estimation.

2.7. Statistical analysis

All experiments in flasks were performed in triplicate, while two parallel samples were taken from batch cultures. The results were expressed as means ± SD. Data were analyzed using Student's *t*-test and *F*-test, and *P* values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Complex mutagenesis for screening non-pigment producing strain

Pigment is an obstacle to pullulan industrial production because it increases pullulan recovery and purification costs (Singh, Saini, & Kennedy, 2009). Hence, a strain deficient in pigment formation for efficient pullulan production is necessary. Complex mutagenesis of *A. pullulans* ATCC 201253 by UV and γ-ray exposure was conducted and the results are listed in Table 1. After treatment, a non-pigment-producing strain of *A. pullulans* SZU 1001 was screened. The mutant showed slight improvement in pullulan production, but produced no pigment during cultivation.

3.2. The single-factor experiments on medium composition

The effects of six nutrients: yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $MgSO_4{\cdot}7H_2O$ and $CaCl_2$ on pullulan production were investigated (Fig. 1). Results of the single-factor design experiments (SFD) showed that $MgSO_4{\cdot}7H_2O$ and $CaCl_2$ had scarcely any significant influences on cell growth, molecular weight or pullulan yield

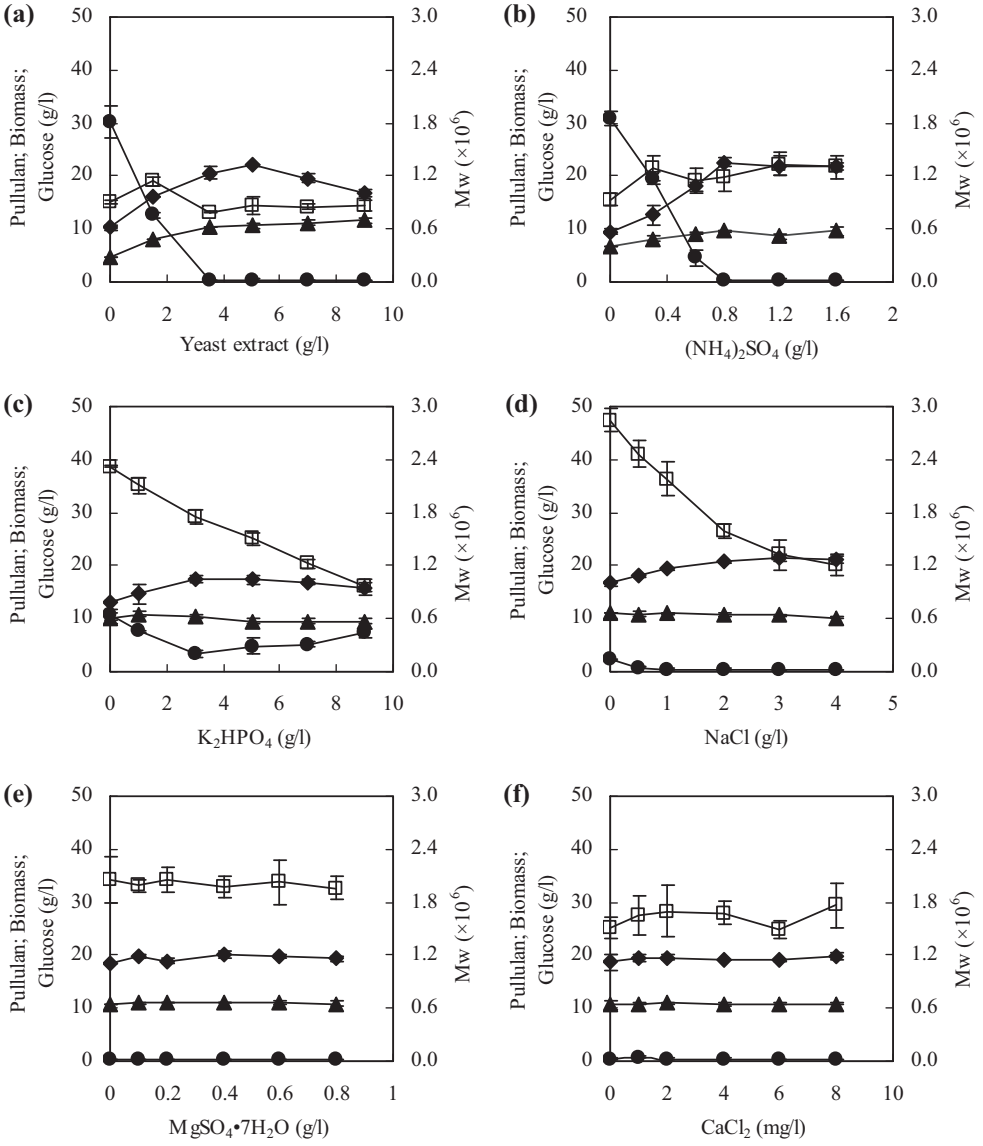

Fig. 1. Effects of nutrients on pullulan production within the single-factor experiments. Biomass (▲), pullulan (♦), residual glucose (●), molecular weight of pullulan (□). Data are shown as mean ± SD ($n = 3$).

with the given concentrations. Conversely, the other four nutrients showed moderate effects on either molecular weight or pullulan yield.

Based on the results depicted in Fig. 1(a) and (b), it was obvious that a lower concentration of nitrogen source as yeast extract and $(NH_4)_2SO_4$ could not satisfy cell growth requirements, and thus resulted in a large amount of residual glucose. A particular concentration of nitrogen seemed necessary to increase pullulan production, while the molecular weight of pullulan experienced limited alteration in the given concentrations. In addition, K_2HPO_4 and NaCl showed the trend in a different way, as depicted in Fig. 1(c) and (d). Pullulan molecular weight decreased dramatically with increased ratios of both salts in the medium, while pullulan production was improved by K_2HPO_4 and/or NaCl increments.

The effect of yeast extract on pullulan yield has been widely investigated, and our results were similar to a previous study (Seo et al., 2004). $(NH_4)_2SO_4$ substantially affected pullulan production, but had only a slight impact on pullulan molecular weight, which was in agreement with former results in the literature (Cheng, Demirci, Catchmark, & Puri, 2011). K_2HPO_4 showed a bell-shaped curve on pullulan production, but it resulted in a decline of pullulan molecular weight (Wiley et al., 1993). NaCl contributed to pullulan production, but here we presented for the first time that NaCl negatively influenced pullulan molecular weight.

3.3. Screening of significant nutrients using FFD

FFD can be used for screening factors from several nutrients that significantly influence pullulan molecular weight and production (Lin et al., 2007), within which a two-level factorial design is most powerful for estimating main effects and interactions. Based on the results of the single-factor experiments, an FFD was performed to search for significant factors within the six nutrients (Table 2). The analysis of variance for the experiment design was calculated and illustrated in Table 3. Significant levels of each medium variable on molecular weight and pullulan yield were determined by t-test. Results indicated that K_2HPO_4 and NaCl had significant effects on the molecular weight of pullulan, while yeast extract and $(NH_4)_2SO_4$ strongly affected pullulan yield.

With the above significant factors, we can seek optimal nutrient combinations in the following study for increasing pullulan

Table 2
Experiments and the results of the fractional factorial design.

Run	Variables and levels[a]						Results		
	A (g/l)	B (g/l)	C (g/l)	D (g/l)	E (g/l)	F (mg/l)	Biomass (g/l)	Yield[b] (%)	Mw ($\times 10^6$)
1	−1 (0.8)	−1 (0.2)	−1 (1.0)	+1 (1.0)	+1 (0.2)	+1 (4.0)	5.92 ± 0.06	20.80 ± 0.76	2.56 ± 0.32
2	+1 (1.6)	−1 (0.2)	+1 (2.0)	−1 (0.5)	+1 (0.2)	−1 (2.0)	8.20 ± 0.46	26.50 ± 1.5	3.37 ± 0.31
3	−1 (0.8)	+1 (0.4)	−1 (1.0)	−1 (0.5)	+1 (0.2)	−1 (2.0)	6.93 ± 0.28	25.60 ± 0.46	2.90 ± 0.11
4	+1 (1.6)	+1 (0.4)	+1 (2.0)	+1 (1.0)	+1 (0.2)	+1 (4.0)	9.25 ± 0.21	40.36 ± 0.22	2.10 ± 0.13
5	−1 (0.8)	−1 (0.2)	+1 (2.0)	+1 (1.0)	−1 (0.1)	−1 (2.0)	6.10 ± 0.09	21.50 ± 0.46	1.91 ± 0.11
6	+1 (1.6)	−1 (0.2)	−1 (1.0)	−1 (0.5)	−1 (0.1)	+1 (4.0)	8.55 ± 0.20	30.80 ± 0.96	3.68 ± 0.15
7	−1 (0.8)	+1 (0.4)	+1 (2.0)	−1 (0.5)	−1 (0.1)	+1 (4.0)	7.28 ± 0.08	28.90 ± 1.74	3.29 ± 0.07
8	+1 (1.6)	+1 (0.4)	−1 (1.0)	+1 (1.0)	−1 (0.1)	−1 (2.0)	8.92 ± 0.30	34.70 ± 1.14	2.42 ± 0.13

[a] A, yeast extract; B, $(NH_4)_2SO_4$; C, K_2HPO_4; D, NaCl; E, $MgSO_4{\cdot}7H_2O$; F, $CaCl_2$.
[b] Yield represented the yield of pullulan on glucose.

Table 3
Analysis results of the experiments with statistical methods (ANOVA).

Factor	df	Mw		Yield of pullulan		Rank of importance	
		Mean square	*p*-Value Prob > *F*	Mean square	*p*-Value Prob > *F*	Mw	Yield of pullulan
Yeast extract	1	1385.13	0.0107	73.96	< 0.0001	3*	1**
$(NH_4)_2SO_4$	1	1103.89	0.0199	62.81	< 0.0001	4*	2**
K_2HPO_4	1	1638.47	0.0064	2.43	0.0505	2**	5
NaCl	1	51446.90	< 0.0001	4.69	0.0115	1**	4*
$MgSO_4{\cdot}7H_2O$	1	130.51	0.3817	0.068	0.7189	6	6
$CaCl_2$	1	970.28	0.0274	4.73	0.0113	5*	3*

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

molecular weight and yield using an orthogonal array design and response surface methodology, respectively.

3.4. Nutrient optimization using an orthogonal array design to increase pullulan molecular weight

The molecular weight of pullulan usually determines the scope of its applications (Ma, Li, Gao, & Hou, 2000). Specifically, pullulan with high molecular weight can be modified and used as drug carrier for disease therapy (Dreher et al., 2006; Mehvar, 2003). Based on the results of SFD and FFD, an $L_9(3^4)$ orthogonal array experiment consisting of yeast extract, $(NH_4)_2SO_4$, K_2HPO_4 and NaCl was carried out, in order to achieve optimal concentrations of each nutrient to increase the molecular weight of pullulan. The cultivation of *A. pullulans* SZU 1001 within the orthogonal array experiments was conducted for 48 h. Statistical analysis of the data using Minitab 16, NaCl was found to be the most important factor influencing the molecular weight of pullulan, followed by yeast extract, K_2HPO_4 and $(NH_4)_2SO_4$ (Table 4). Based on the analysis of the orthogonal experiments in Fig. 2, the optimal concentrations of yeast extract, $(NH_4)_2SO_4$, K_2HPO_4 and NaCl for high molecular weight were 1.0 g/l, 0.3 g/l, 1.0 g/land 0.0 g/l, respectively. Results from Fig. 2 also revealed a similar tendency to those from the single-factor experiments, which were shown in Fig. 1(a–d).

A molecular weight of up to 5.32×10^6 was achieved in the orthogonal experiment. This level has never been reported in previous studies on pullulan production with glucose as carbon source (Chen, Wu, & Pan, 2012; Israilides et al., 1994; Lee et al., 1999; Prasongsuk et al., 2007). Thus, an optimal medium for high molecular weight of pullulan should be summarized as: 50 g/l glucose, 1.0 g/l yeast extract, 0.3 g/l $(NH_4)_2SO_4$, 1.0 g/l K_2HPO_4 and 0.2 g/l $MgSO_4{\cdot}7H_2O$.

3.5. Enhanced pullulan production using response surface methodology

Increased pullulan production and yield were the main objectives during industrial processing optimization (Jiang, 2010; Sena, Costelli, Gibson, & Coughlin, 2006). In this study, the effect of nutritional factors on pullulan yield was investigated using response surface methodology. Based on results derived from single-factor experiments and FFD, a steep ascent experiment was carried out to

Table 4
Experimental results and analysis of the $L_9(3^4)$ orthogonal experiments for pullulan production.

Run	Variables and levels				Results		
	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	K_2HPO_4 (g/l)	NaCl (g/l)	Biomass (g/l)	Pullulan (g/l)	Mw ($\times 10^6$)
1	3 (3.0)	1 (0.3)	3 (5.0)	2 (1.0)	10.30 ± 0.13	15.60 ± 0.45	1.63 ± 0.04
2	1 (1.0)	2 (0.5)	2 (3.0)	2 (1.0)	7.65 ± 0.18	14.45 ± 0.53	2.74 ± 0.07
3	3 (3.0)	3 (0.8)	2 (3.0)	1 (0)	11.68 ± 0.04	20.48 ± 0.11	2.61 ± 0.24
4	2 (2.0)	1 (0.3)	2 (3.0)	3 (2.0)	7.93 ± 0.13	14.65 ± 0.09	1.50 ± 0.03
5	2 (2.0)	2 (0.5)	3 (5.0)	1(0)	9.75 ± 0.05	16.25 ± 0.35	2.93 ± 0.28
6	2 (2.0)	3 (0.8)	1 (1.0)	2 (1.0)	10.45 ± 0.28	14.63 ± 0.32	2.74 ± 0.16
7	1 (1.0)	1 (0.3)	1 (1.0)	1 (0)	6.33 ± 0.19	9.15 ± 0.54	5.32 ± 0.02
8	3 (3.0)	2 (0.5)	1 (1.0)	3 (2.0)	9.93 ± 0.13	22.30 ± 1.54	1.62 ± 0.06
9	1 (1.0)	3 (0.8)	3 (5.0)	3 (2.0)	8.48 ± 0.33	19.65 ± 0.79	1.69 ± 0.12
$R_{Biomass}$	3.03	1.83	0.67	0.62			
R_{Yield}	5.18	5.70	1.37	3.53			
R_{Mw}	0.73	0.25	0.65	1.08			

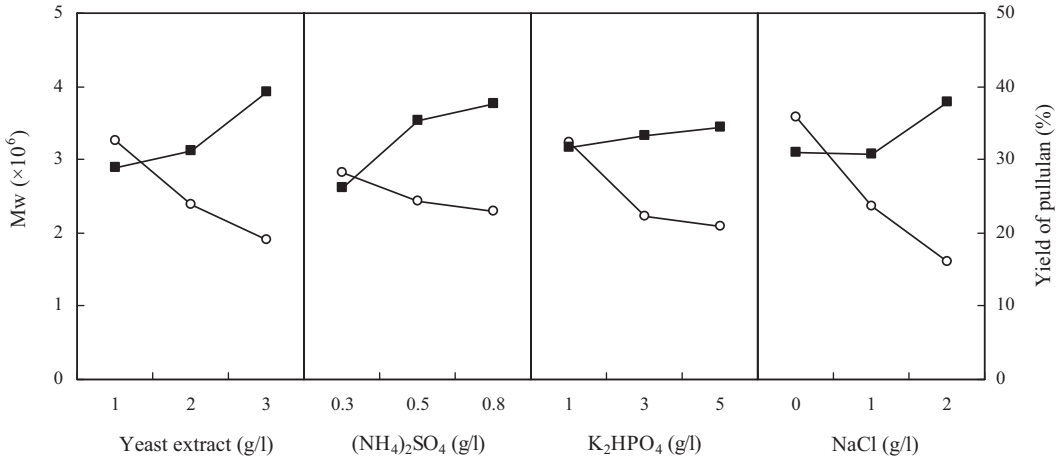

Fig. 2. The intuitive analysis on the main factors effecting the yield (■) and molecular weight (○) of pullulan in the $L_9(3^4)$ orthogonal array design.

Table 5
The design and results of the steepest ascent experiment.

Steps	Level of nutrients		Response
	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	Yield of pullulan (%)
1	0.8	0.24	20.40 ± 1.20
2	1.2	0.32	28.30 ± 0.70
3	1.6	0.40	37.50 ± 1.50
4	2.0	0.48	47.80 ± 0.62
5	2.4	0.56	50.10 ± 0.42
6	2.8	0.64	45.76 ± 0.22

Table 6
The experiment design and results of the central composite design.

Run	Level of nutrients		Response
	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	Yield of pullulan (%)
1	0(2.5)	0(0.6)	48.90 ± 1.32
2	−1.414 (1.1)	0(0.6)	34.66 ± 1.48
3	−1 (1.5)	+1 (0.8)	39.00 ± 3.40
4	+1 (3.5)	+1 (0.8)	44.40 ± 2.62
5	+1 (3.5)	−1 (0.32)	44.30 ± 1.06
6	+1.414 (3.9)	0(0.6)	47.10 ± 1.80
7	0(2.5)	0(0.6)	47.40 ± 1.46
8	0(2.5)	+1.414 (0.88)	42.60 ± 0.84
9	0(2.5)	0(0.6)	48.00 ± 2.24
10	0(2.5)	−1.414 (0.32)	41.90 ± 0.46
11	0(2.5)	0(0.6)	48.30 ± 1.76
12	−1 (1.1)	−1 (0.4)	35.10 ± 1.50
13	0(2.5)	0(0.6)	47.40 ± 2.04

determine the center point necessary for central composite design (CCD). The experiments were performed according to the design in Table 5, and the center point was found where yeast extract was 2.4 g/l and $(NH_4)_2SO_4$ was 0.56 g/l. The concentrations of the two factors were adjusted to 2.5 g/l and 0.6 g/l, respectively, in the actual implementation process for calculation convenience. The factors and levels involved in CCD are illustrated in Table 6, which were verified through the preceding experiments. Response surface methodology was used to determine the optimal levels of the two nutritional factors to optimize pullulan yield. Thirteen experiments were carried out using CCD and results are shown in Table 6. After regression analysis, a quadratic polynomial model was obtained as follows to fit the CCD experiment results:

$$\text{yield of pullulan} = -24.306 + 26.557A + 111.899B - 4.589AB - 3.933A^2 - 81.139B^2 \quad (1)$$

where A is the yeast extract concentration (g/l) and B is the $(NH_4)_2SO_4$ concentration (g/l). The statistical significance of this regressive equation was verified through ANOVA for the response surface quadratic model, and the results are summarized in Table 7. The model was significant at a level of 5%, as demonstrated by the F-value, while the lack of fit was not significant. The predicted R^2 value of 0.8456 was reasonable with the adjusted R^2 value of 0.8891, which implied that the model was adequate for prediction. 3D response surface plots based on the model are shown in Fig. 3. The optimal concentrations for maximum pullulan yield based on the model were calculated as 3.0 g/l yeast extract and 0.6 g/l $(NH_4)_2SO_4$, which predicts a concentration of about 25 g/l of pullulan. Hence, an optimum medium for efficient pullulan production could be achieved using 50 g/l glucose, 3.0 g/l yeast extract, 0.6 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 1.0 g/l NaCl and 0.2 g/l MgSO4·7H_2O.

3.6. Batch pullulan production with optimized media

To validate the predictions of the optimized media from the orthogonal array design (Medium-Mw) and response surface

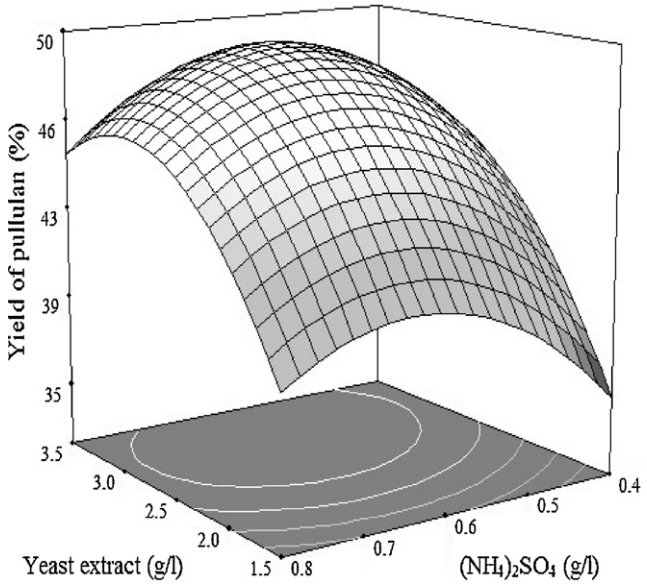

Fig. 3. The response surface plot for high yield of pullulan by *A. pullulans* SZU 1001 showing the effects of yeast extract and $(NH_4)_2SO_4$ on pullulan production.

Table 7
Analysis of variance for the experimental results of the central composite design.

Source	Sum of squares	df	Mean square	*F*-value	*p*-Value Prob > *F*	Significance
A-Yeast extract	331.58	1	331.58	121.78	<0.0001	**
B-$(NH_4)_2SO_4$	7.62	1	7.62	2.80	<0.0001	**
AB	9.12	1	9.12	3.35	0.1092	
A^2	194.68	1	194.68	71.50	<0.0001	**
B^2	132.57	1	132.57	48.69	<0.0001	**
Model	581.43	5	116.29	42.71	<0.0001	**
Lack of fit	11.11	3	3.70	1.45	0.2621	
Pure error	46.06	18	2.56			
Total	638.61	26				

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

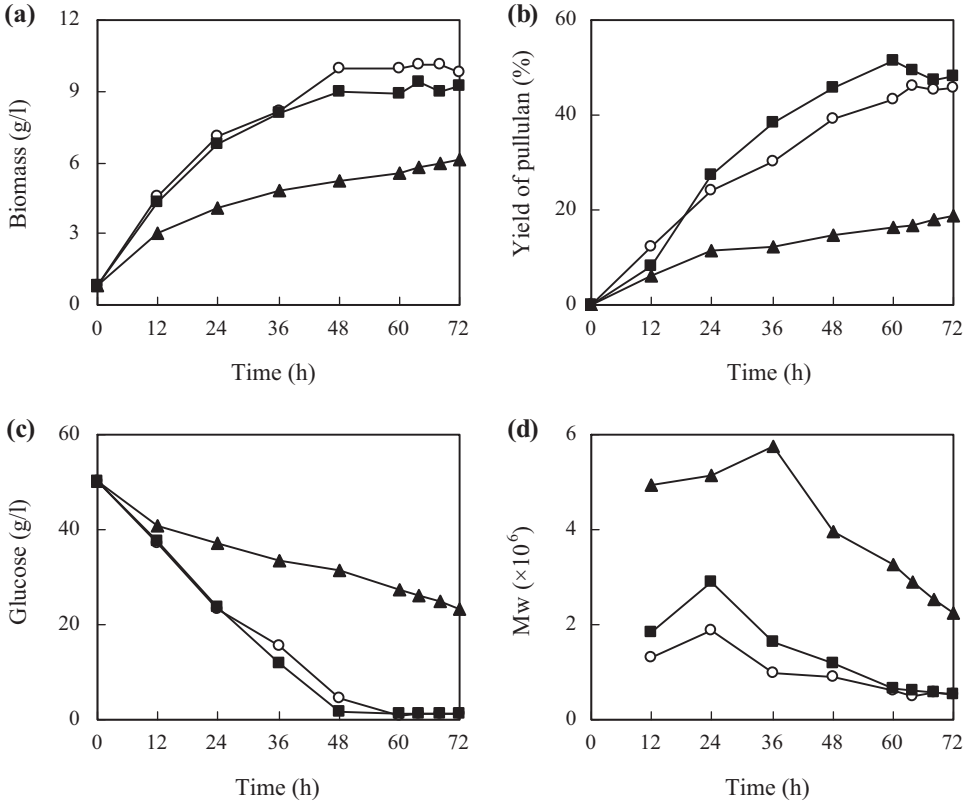

Fig. 4. Time-course of batch pullulan fermentation by *A. pullulans* SZU 1001 using different media. Medium-control (○) consisted of 50 g/l glucose, 2.5 g/l yeast extract, 5.0 g/l K_2HPO_4, 0.6 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 1.0 g/l NaCl; Medium-Mw (▲) consisted of 50 g/l glucose, 1.0 g/l yeast extract, 0.3 g/l $(NH_4)_2SO_4$, 1.0 g/l K_2HPO_4 and 0.2 g/l $MgSO_4{\cdot}7H_2O$; and Medium-yield (■) consisted of 50 g/l glucose, 3.0 g/l yeast extract, 0.6 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 1.0 g/l NaCl and 0.2 g/l $MgSO_4{\cdot}7H_2O$.

methodology (Medium-yield), the initial medium (Medium-control) was selected as the control for batch pullulan fermentation using *A. pullulans* SZU 1001. As shown in Fig. 4, comparisons between the three media effectively confirmed the preceding experimental results. Medium-control led to a higher biomass, and Medium-Mw and Medium-yield resulted in increased molecular weight and pullulan production, respectively. Glucose consumption and cell growth rates were much lower with the Medium-Mw than the other two media during cultivation, but could increase pullulan molecular weight as high as 5.74×10^6 at 36 h, which was an increase of 97.25% compared to the control medium.

According to the results in Fig. 4(b) and (d), the highest molecular weight of pullulan could be obtained only before the highest pullulan production was achieved. Molecular weight tendencies are shown in Fig. 4, which were consistent with other reports (Seo et al., 2004; Wiley et al., 1993). Medium-Mw resulted in pullulan with a tremendously high molecular weight, which made pullulan production with a specific Mw feasible. The maximum pullulan output of 25.65 g/l was realized using the Medium-yield, which was in agreement with the predicted value of 25 g/l using the response surface methodology. The yield of pullulan on glucose reached 51.30% under batch fermentation in only 60 h, which increased by 11.04% as compared to the control, and thus made efficient pullulan production possible using *A. pullulans* SZU 1001.

4. Conclusions

Pullulan continues to attract increasing attention owing to its expanding application potential in the food and cosmetic industries and for environment treatment, etc. Pullulan molecular weight and yield are important indices during pullulan fermentation using a pigment-free mutant of *A. pullulans* SZU 1001, which was screened by complex mutagenesis with UV and γ-ray on the parent strain of *A. pullulans* ATCC 201253. Based on single-factor design and

the FFD results, the most important nutrients that influence pullulan molecular weight and yield were chosen for further study. With the aim of increasing pullulan molecular weight, an $L_9(3^4)$ orthogonal array design was introduced to optimize medium composition, which resulted in a combination of nutrients: 50 g/l glucose, 1.0 g/l yeast extract, 0.3 g/l $(NH_4)_2SO_4$, 1.0 g/l K_2HPO_4 and 0.2 g/l $MgSO_4{\cdot}7H_2O$. Under this optimized condition, a maximum molecular weight of 5.74×10^6 was obtained through batch pullulan fermentation with *A. pullulans* SZU 1001. Furthermore, a statistical approach of response surface methodology was used to optimize nutrients to increase pullulan yield. After analysis of central composite design results, an optimal medium for efficient pullulan production was summarized as follows: 50 g/l glucose, 3.0 g/l yeast extract, 0.6 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 1.0 g/l NaCl and 0.2 g/l $MgSO_4{\cdot}7H_2O$, which resulted in 25.65 g/l pullulan with a yield of 51.30% of pullulan on glucose within 60 h. Herein, we presented feasible approaches based on statistical analyses for efficient pullulan production either with high molecular weight or high yield. By using the optimized media, individual pullulan production for special utilization in medicine will be soon be realized at a relatively low cost.

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