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Application of statistical experimental design for optimization of downstream process for recovery of pullulan produced by *Aureobasidium pullulans* HP-2001

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Abstract−The optimal conditions of the downstream process for recovery of pullulan produced by *Aureobasidium pullulans* HP-2001 were examined using response surface method (RSM). The optimal amount of diatomite in filter press and the optimal flow rate in a continuous flow centrifuge for removal of cells from the culture broth of *A. pullulans* HP-2001 were found to be 5.0% (v/v) and 2.0 L/min. Based on central composite design (CCD) experiments and analysis of variance (ANOVA) indicated that the optimal conditions for recovery of pullulan from the supernatant by precipitation were the volume ratio of ethanol (or isopropanol) to supernatant of 3.0 : 1.0, the reaction time of 29.5 h, and the reaction temperature of 20.2 °C. The expected maximal recovery yields of pullulan using ethanol and isopropanol under optimized conditions were 79.2 and 85.5%, respectively.

Key words: Pullulan, Down-stream Process, Filter Press, Continuous Flow Centrifuge, Response Surface Method

INTRODUCTION

Pullulan is a water-soluble microbial polysaccharide that can be produced in large quantities by fermentation of *Aureobasidium pullulans* [1]. The regular alternation of α-1, 4 and α-1, 6 bonds of maltotriose and maltotetraose units results in two distinct properties of structural flexibility and enhanced solubility [2]. Due to these properties, pullulan provides highly viscous solutions at relatively low concentrations and can be used to make an oxygen-impermeable membrane [3]. Pullulan has a wide range of commercial and industrial applications in many fields, such as food industry, pharmaceuticals manufacturing, health care and cosmetic industry [1].

The cost for downstream in the fermentation based process normally accounts for more than 60% of the total cost for production [4]. The development of a simplified and cost-effective process for downstream of microbial polysaccharides is a constant major challenge for their commercialization. However, previous investigations on pullulan have been directed toward its fermentation including optimizations of media, initial pH, culture temperature, agitation speed, aeration rate, and inner pressure of bioreactors [5,6]. Studies on downstream, including removal of cells and recovery of pullulan from supernatants of culture broths, have not been carried out except for those related to basic information such as solvents like ethanol and isopropanol to precipitate pullulan in the culture broths [7,8]. Especially, there has been no report on simultaneous effects of solvents, reaction time, and temperature on recovery of pullulan from supernatant after removal of cells.

The optimization of conditions for recovery of pullulan by classical methods involving the change of one variable at a time is extremely time-consuming and expensive when a large number of variables are considered [9]. As a powerful statistical and mathematical tool, response surface methodology (RSM) provides important information regarding the optimum level of each variable along with its interaction with other variables and their influences on production [10]. In this study, effects of diatomite in a filter press and the flow rate of a continuous centrifuge on removal of cells from a culture broth of *A. pullulan* HP-2001 were examined. The ratio of ethanol (or isopropanol) to supernatant, reaction time, and temperature for recovery of pullulan from the supernatant after removal of cells from culture broths of *A. pullulan* HP-2001 were also optimized using RSM.

MATERIALS AND METHODS

1. Bacterial Strain and Medium

A strain of *Aureobasidium pullulans* HP-2001, a UV-induced mutant of *A. pullulans* ATTC 42023, was transferred monthly to fresh nutrient agar medium [5]. The medium for the cell growth contained 20 g/L glucose, 0.25 g/L yeast extract, 2.5 g/L K_2HPO_4, 0.5 g/L NaCl, 0.05 g/L $MgSO_4 \cdot 7H_2O$, and 0.3 g/L $(NH_4)_2SO_4$. Glucose was autoclaved separately and added to the medium in aseptic conditions.

2. Mass Production of Pullulan

Starter cultures were prepared by transferring cells from agar slants to 50 mL of medium in 250 mL Erlenmeyer flasks. These cultures were incubated at 30 °C and for 24 h under aerobic conditions and used to inoculate 150 mL of medium in 500 mL Erlenmeyer flasks. Cultures were incubated for 24 h under the same conditions used in preparing the starter cultures and used to inoculate 4 L medium

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in a 7 L bioreactor (Ko-Biotech Co., Inchon, Korea). After 24 h of cultivation in 7 L bioreactors with an agitation speed of 400 rpm and an aeration rate of 1.0 vvm, these cultures were used to inoculate 70 L medium in a 100 L bioreactor ((Ko-Biotech Co., Inchon, Korea). Carbon and nitrogen sources for batch fermentation were 50.0 g/L glucose and 2.5 g/L yeast extract. Initial pH of the medium and temperature were 6.0 and 25 °C. The agitation speed and aeration rate of a 100 L bioreactor were 250 rpm and 1.0 vvm. Agitation was provided by three six-flat-blade impellers in a 100 L bioreactor. The inner pressure of a 100 L bioreactor was 0.04 kgf/cm^2. Inoculum sizes of batch fermentations for production of the pullulan by *A. pullulans* HP-2001 were 5% (v/v).

Culture broths after 96 h of cultivation were centrifuged at 12,000 ×g for 15 min at 4 °C to remove cells. The supernatant was added to two volumes of isopropyl alcohol, left to stand overnight at 4 °C to precipitate pullulan, and centrifuged at 12,000 ×g for 15 min to separate the precipitate. The precipitated material was repeatedly washed with isopropyl alcohol and dissolved in deionized water, and dialyzed against deionized water (DW) using dialysis tubing with a molecular weight cut off of 12,000~14,000 Da. After dialysis for 2-3 days with 4-5 changes of DW, the dialyzed solution was lyophilized.

3. Experimental Design and Optimization

The volume ratio of ethanol (or isopropanol) to supernatant (X_1), reaction time (X_2, h), and temperature (X_3, °C) were chosen as the independent variables. Recovered pullulan (Y, g/L) was used as a dependent output variable. The total number of experiments was 20 (=2^k+2k+6), where k is the number of independent variables [11]. And each variable was designated as −1, 0, and 1, respectively. The interrelationships of the variables were determined by fitting the second degree polynomial equation to data obtained from 20 experiments using mean values of the triplicates of each experiment conducted trice at different occasions. The maximum values of the yield were taken as the responses of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The minimum and maximum range of variables and the full experimental plan with respect to their actual and coded values are shown in Table 1. A multiple regression analysis of the data was carried out with the statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA) and the second order polynomial Eq. (1) that defines predicted response (Y) in terms of the independent variables (X_1, X_2, and X_3) was obtained:

$$Y_1 \text{ (or } Y_2) = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 \quad (1)$$

Table 1. Process variables used central composite design (CCD) with actual factor levels corresponding to coded factor levels

Variables	Symbol	Coded levels		
		−1	0	1
Ratio of ethanol (or isopropanol) to supernatant	X_1	1.0 : 1.0	2.0 : 1.0	3.0 : 1.0
Time (h)	X_2	6	18	30
Temperature (°C)	X_3	18	24	30

Y_1 or Y_2 is the amount of pullulan precipitated by ethanol or isopropanol, respectively. Where X_1, X_2, and X_3 are input variables; A_0 is constant; A_1, A_2, and A_3 are linear coefficients; A_{12}, A_{13}, and A_{23} are cross-product coefficients; A_{11}, A_{22}, and A_{33} are quadratic coefficients. Combinations of factors (such as X_1X_2) represent an interaction between the individual factors in that term. Then the response is a function of the levels of factors.

4. Analytical Methods

Dry cells weight and concentration of pullulan were measured as described in the previous report [5]. A standard curve for quantitation of pullulan was prepared from authentic pullulan (Sigma-Aldrich, St. Louis, USA). Reducing sugar content in the culture broth was determined by the dinitrosalicylic acid (DNS) method [12]. DNS reagent (1 mL) was mixed with 0.3 mL of the culture broth, and the mixture was placed in a boiling water bath for 15 min. After the mixture was cooled to room temperature, the concentration of reducing sugar was examined at 550 nm with a spectrophotometer (Unicam Co., Helios Delta, UK).

RESULTS AND DISCUSSION

1. Mass Production of Pullulan by *A. pullulans* HP-2001

Pullulan was produced by *A. pullulans* HP-2001 in a 100 L bioreactor. The pH of the medium rapidly decreased until 24 h after cultivation, then was maintained at around 3.7, as shown in Fig. 1. The concentration of the dissolved oxygen in the medium dramatically decreased and then reached around 0% in the late stage of the log phase. After a limited time with a shortage of dissolved oxygen, it rose gradually until the final stage of cultivation. A certain period of time with a shortage of dissolved oxygen in the medium has been reported to be necessary to enhance the production of pullulan [5]. After cell growth ceased, the production of pullulan by *A. pullulans* HP-2001 continued until 72 h of cultivation. Production of pullulan from 50.0 g/L glucose and 2.5 g/L yeast extract as carbon and nitrogen sources was 18.7 g/L during 96 h of cultivation in a 100 L bioreactor. Production of pullulan from 30.0 g/L sucrose and 2.5 g/L yeast extract as carbon and nitrogen sources by *A. pullulan* was 17.5 g/L and its conversion rate was 35.0% [6]. Maximal pro-

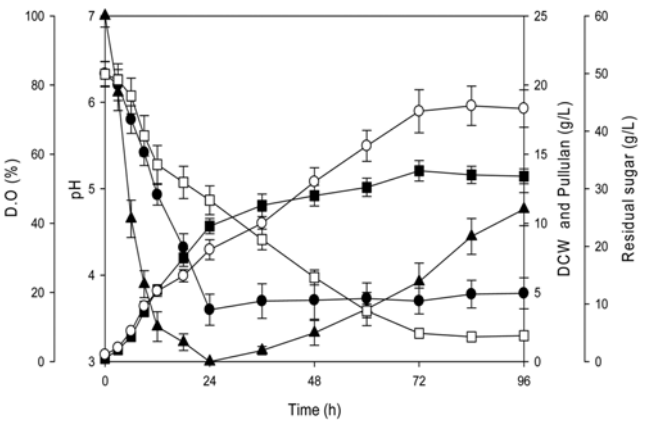

Fig. 1. Cell growth and the production of pullulan by *A. pullulans* HS-2001 in a 100 L bioreactor (●, pH; ■, dry cells weight; ▲, dissolved oxygen; ○, pullulan; and □, residual sugars).

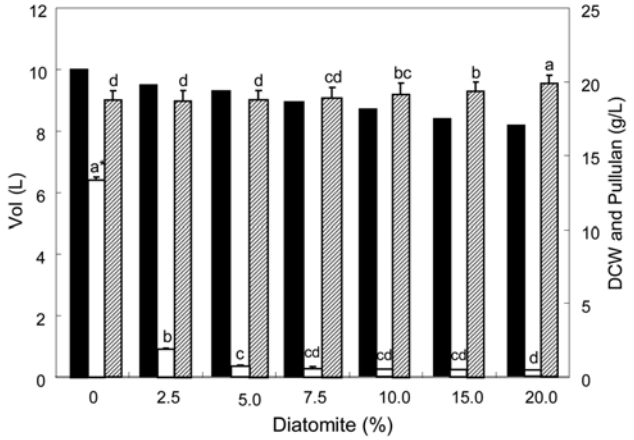

Fig. 2. Effect of diatomite in a filter press on removal of cells and recovery of pullulan from the culture broth of *A. pullulans* HP-2001 (■, volume of culture broth after filtration; □, dry cells weight; and ▨, pullulan). Different letters above bars mean that each value with a different letter is significantly different at $p<0.05$.

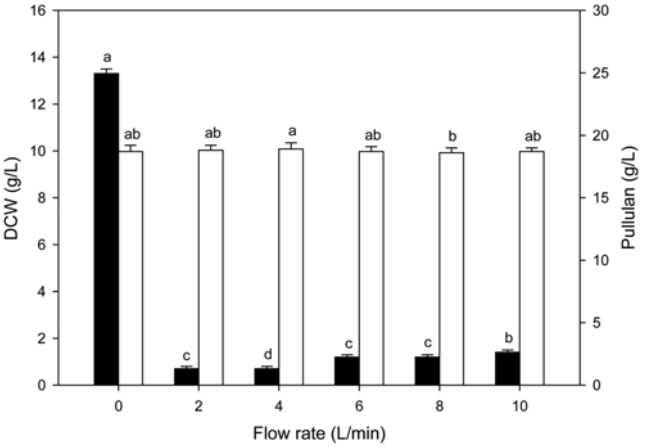

Fig. 3. Effect of flow rate in a continuous flow centrifuge on removal of cells and recovery of pullulan from the culture broth of *A. pullulans* HP-2001 (■, dry cells weight and □, pullulan).

duction of pullulan from 100.0 g/L glucose by *A. pullulan* was reported to be 36.9 g/L; however, the concentration of residual sugar after fermentation was relatively high, which interrupted to recover pullulan from culture broth [5]. Continuous culture of *A. pullulans* was reported to enhance production of pullulan as well as its conversion rate [6].

2. Effect of Diatomite in Filter Press on Removal of Cells

The effect of diatomite on removal of cells and recovery of pullulan from culture broths of *A. pullulans* HP-2001 was examined in a filter press (Plate & Frame Type Filter Press, Korea filter, Seoul, Korea). The filter press used had two chambers and the filter area and volume of each chamber were 0.11 m^2 and 1.7 L. The culture broths were 10 L in volume and amount of diatomite mixed with culture broths ranged from 0.0 to 20.0%. Removal of cells from culture broth increased with increased amounts of diatomite; however, the recovery yield of pullulan decreased due to reduced volume of filtrate, as shown Fig. 2. Significance of each value was analyzed by DPS software version 3.01 (DPS Co., Middlesex, UK). The highest recovery yield of pullulan from culture broth was 94.4% when 2.5% (w/v) diatomite was mixed with culture broth; however, the removed cells from culture broth were relatively low. Based on the amount of removed cells and the recovery yield of pullulan, the optimal concentration of diatomite for removal of cells from the culture broth of *A. pullulans* HP-2001 was chosen to be 5.0%. In this condition, 94.4% of cells were removed from the culture broth and the recovery yield of pullulan was 92.8%.

For mass production of microbial exopolysaccharides, cells are usually removed first, either by the filtration with a filter press or by centrifugation with a continuous flow centrifuge at the end of fermentations [13]. Due to its unique physical properties, diatomite has been commonly used in water purification, filtration of commercial fluids, clarifications of liquors and juices, and separation of various oils and chemicals [14,15].

3. Effect of Flow Rate of Continuous Centrifuge on Removal of Cells

The effect of flow rate on removal of cells and recovery of pullulan from the culture broth of *A. pullulans* HP-2001 was also examined using a continuous flow centrifuge (Tubular Type Centrifuge, Kokusan Chemical Co., Tokyo, Japan). The continuous flow centrifuge had a bowl with a capacity of 9.0 L and a diameter of 12.2 cm. Its rotational speed and relative centrifugal force were fixed to be 12,000 rpm and 11,500 ×g, respectively. The flow rate into the continuous flow centrifuge ranged from 0.0 to 10.0 L/min. Removal of cells from culture broth decreased with increased flow rates; however, the recovery yield of pullulan was nearly constant, as shown in Fig. 3. The highest removal rate of cells was 95.0% when the flow rate of the culture broth through the continuous flow centrifuge was 0.2 L/min. Based on the amount of removed cells and the recovery yield of pullulan, the optimal flow rate of the culture broth through a continuous flow centrifuge for removal of cells from the culture broth was decided to be 2.0 L/min.

Centrifugation is a key step in the recovery process of most biological products [16,17]. The use of a continuous flow centrifuge can overcome the loss of culture broth or supernatant during removal of cells using filtration with a filter press. The continuous flow centrifugation offered relatively high recovery equivalent to cartridge filtration, with improved economy and ease of use compared to the filter-based process for removal of cells from the culture broth [18,19].

4. Effects of Ethanol, Reaction Time, and Temperature on Recovery of Pullulan

We used response surface methodology (RSM) to improve recovery yield of pullulan from the supernatant after removal of cells by comparing different levels of several factors that were reported to have more influence on recovery of pullulan by precipitation. The results of central composite design (CCD) experiments consisted of experimental data for studying the effects of three independent variables: volume ratio of ethanol to the supernatant, reaction time, and temperature on recovery of pullulan by precipitation are shown in Table 2. The analysis of variance (ANOVA) indicated that the model terms of X_1, X_2, and X_3^2 were highly significant ("probe> F" less than 0.001), and X_3 and X_3^2 were significant ("probe>F" less than 0.0500). However, the interactive effects of X_1X_2, X_1X_3, and X_2X_3 were not significant, as shown in Table 3. It means that

Table 2. Central composite design and determined response values (Y_1 and Y_2 were obtained using ethanol or isopropanol for recovery of pullulan by precipitation, respectively)

Run	X_1	X_2	X_3	Y_1 (g/L)	Y_2 (g/L)
1	3.0 : 1.0	18	24	14.97	15.97
2	3.0 : 1.0	30	30	15.17	16.27
3	2.0 : 1.0	18	30	14.73	15.93
4	3.0 : 1.0	30	18	15.23	16.33
5	1.0 : 1.0	18	18	14.20	15.50
6	1.0 : 1.0	30	30	14.27	15.50
7	1.0 : 1.0	6	18	14.10	15.43
8	2.0 : 1.0	6	18	14.70	15.93
9	2.0 : 1.0	18	30	14.73	15.93
10	1.0 : 1.0	30	18	14.33	15.56
11	1.0 : 1.0	6	30	14.03	15.37
12	2.0 : 1.0	30	18	14.93	16.07
13	3.0 : 1.0	30	30	15.17	16.27
14	3.0 : 1.0	6	30	14.93	16.13
15	1.5 : 1.0	24	24	14.33	15.40
16	1.0 : 1.0	30	30	14.27	15.50
17	3.0 : 1.0	6	18	15.00	16.20
18	3.0 : 1.0	6	30	14.93	16.13
19	1.0 : 1.0	6	30	14.03	15.37
20	2.0 : 1.0	6	24	14.57	15.63

Table 3. Parameter estimates and analysis of variance using ethanol for recovery of pullulan by precipitation

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Model	9	3.11	0.35	294.98	<0.0001
X_1	1	2.72	2.72	2325.72	<0.0001
X_2	1	0.18	0.18	154.94	<0.0001
X_3	1	0.02	0.02	15.36	0.0029
X_1 X_2	1	0.00	0.00	0.03	0.8638
X_1 X_3	1	0.00	0.00	0.02	0.8973
X_2 X_3	1	0.00	0.00	0.13	0.7214
X_1^2	1	0.07	0.07	57.35	<0.0001
X_2^2	1	0.00	0.00	1.00	0.3400
X_3^2	1	0.04	0.04	35.00	0.0001
Error	5	0.00	0.00	-	-
Total	19	3.12	-	-	-

the volume ratio of ethanol to supernatant and reaction temperature have important effects on recovery of pullulan by precipitation. Multiple regression analysis of the experimental data gave the following second-order polynomial equation:

$$Y_1=14.62+0.46X_1+0.11X_2-0.034X_3+1.734\times10^{-3}X_1X_2-1.321\times10^{-3}X_1X_3+3.387\times10^{-3}X_2X_3-0.14X_1^2+0.021X_2^2+0.14X_3^2 \quad (2)$$

Where Y is the response, that is, recovered pullulan, and X_1, X_2, and X_3 are the coded values of the test variables - volume ratio of ethanol to the supernatant, reaction time, and temperature, respectively. After the neglect of insignificant terms (on the basis of "probe >F" which are more than 0.05), the model Eq. (2) was modified to

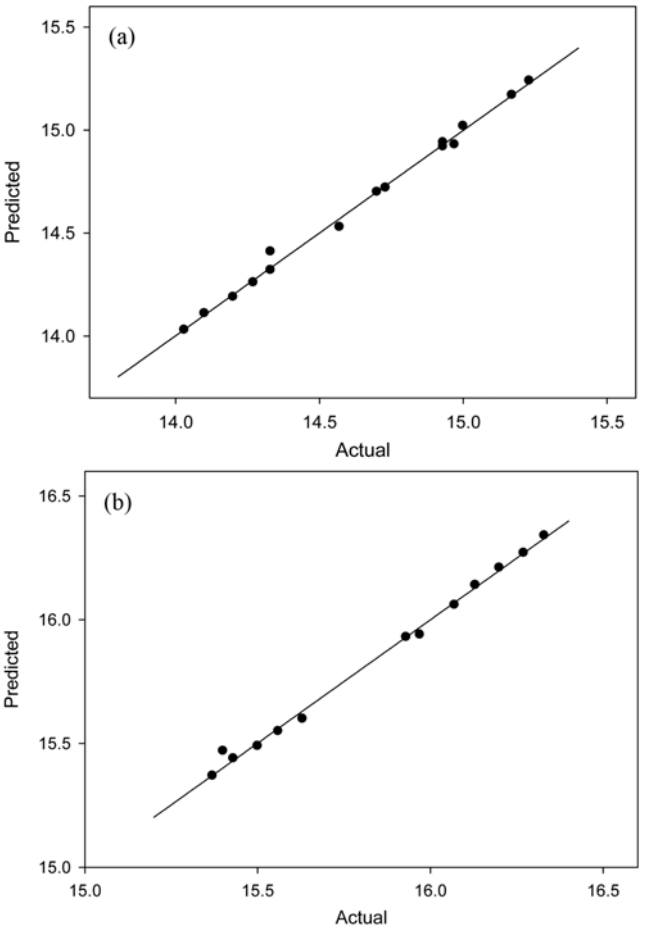

Fig. 4. Distribution of experimentally determined values versus statistically predicted ones using ethanol (a) and isopropanol (b) for recovery of pullulan.

reduced the fitted model Eq. (3):

$$Y_1=14.62+0.46X_1+0.11X_2-0.03X_3-0.14X_1^2+0.15X_3^2 \quad (3)$$

The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.9962. The model can explain 99.62% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9929) is also very high to advocate for a high significance of the model [20,21]. The Pred. R^2 of 0.9846 is in reasonable agreement with Adj. R^2 of 0.9929. The model *F*-value of 294.98 implied that the model was significant. There is only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. From the statistical results obtained, the above models were shown to be adequate to predict the recovery of pullulan using ethanol within the range of variables studied. The optimal conditions extracted by Design Expert Software were the ratio of ethanol to supernatant of 3.0 : 1.0, reaction time of 29.5 h, and temperature of 20.2 °C. The maximum recovered pullulan of 15.13 g/L was predicted by the model. The recovery yield of pullulan from the supernatant was 79.2%.

The experimental determined values were in close agreement with the statistically predicted ones, confirming the model's authenticity, as shown in Fig. 4. The points clustered around the diagonal line, which indicated the good fit of the model. The three-dimen-

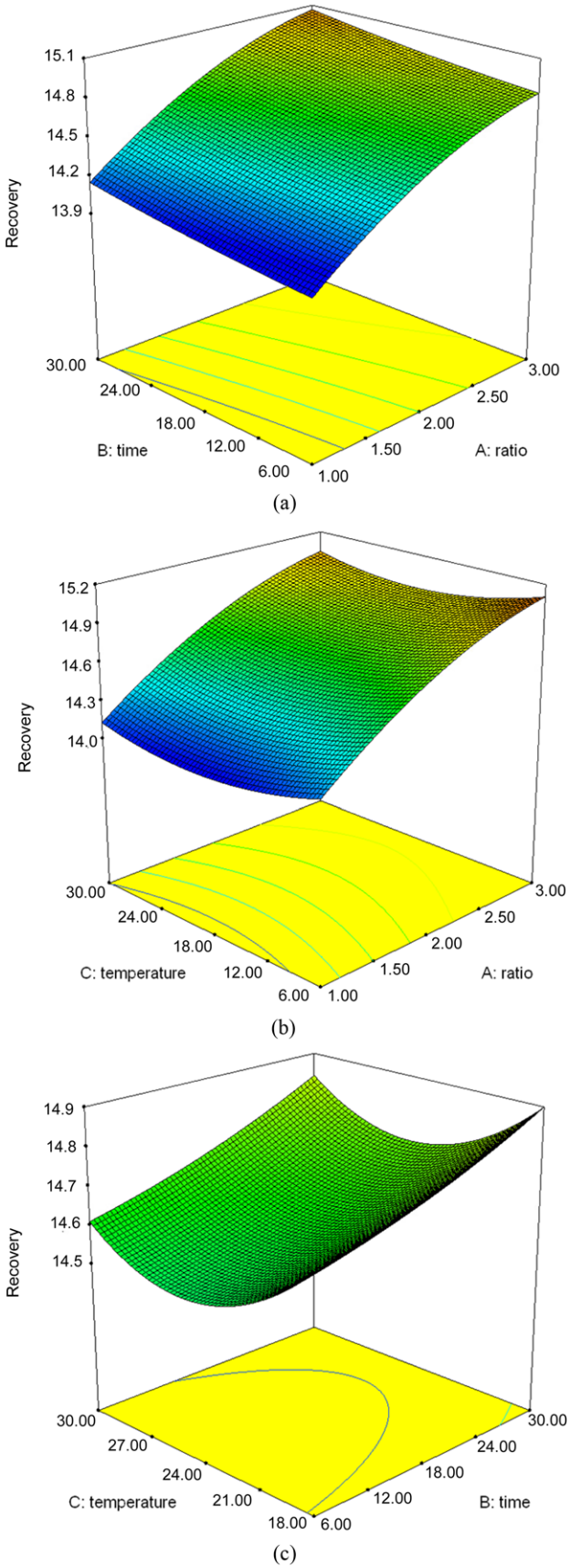

Fig. 5. Three-dimensional contour plots of three factors on recovery of pullulan by ethanol. When the two factors were plotted, the other one factor was set at middle-level values.

sional response surface was generated to study the interaction among three factors tested and to visualize the combined effects of factors on the response of recovery of pullulan by ethanol, as shown Fig. 5. When the effect of two factors was plotted, the other one factor was set at the coded value zero, which was a volume ratio of ethanol to supernatant of 2.0 : 1.0, a time of 18 h, and a temperature of 24 ºC. The combined effects of each pair of factors demonstrated that recovery of pullulan considerably increased with the increased volume ratio of ethanol to supernatant, indicating that the volume ratio of ethanol to supernatant has a significant effect on the responses.

After removal of cells from the culture broth either by filtration or centrifugation, further purification may include precipitation using water-miscible non-solvents such as acetone, ethanol, and isopropanol [13]. Various fraction methods for purification of microbial polysaccharides are mainly based on the difference of their physico-chemical characteristics such as solubility in the suitable solvent mixture, charge density, and molecular mass [22]. Recovery of pullulan from supernatants after removal of cells with various solvents such as ethanol, isopropyl alcohol, and tetrahydrofuran has been carried out [23]. Among methanol, ethanol, isopropanol, ethyl methyl ketone, tetrahydrofuran, and acetone, ethanol was reported to be the most efficient for precipitation of pullulan [7,17]. The optimal reaction time for precipitation of pullulan with two volumes of ethanol at 4 ºC was reported to be from 1 to 6 h [24].

5. Effect of Isopropanol, Reaction Time, and Temperature on Recovery of Pullulan

Response surface methodology (RSM) was also used to improve the recovery yield of pullulan from the supernatant using isopropanol. The results of CCD experiments consisted of experimental data for studying the effects of three independent variables - ratio of isopropanol to supernatant, reaction time, and temperature on precipitation of pullulan. The ANOVA indicated that the model terms of X_1, X_2, X_1^2, and X_3^2 were also highly significant ("probe>F" less than 0.001), and the interactive effects of X_1X_2, X_1X_3, and X_2X_3 were not significant, as shown in Table 4. It means that the volume ratio of isopropanol to supernatant and reaction temperature have important effects on recovery of pullulan by precipitation. Multiple regression analysis of the experimental data gave the following second-

Table 4. Parameter estimates and analysis of variance using isopropanol for recovery of pullulan by precipitation

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Model	14	2.26	0.25	332.90	<0.0001
X_1	1	1.98	1.98	2620.98	<0.0001
X_2	1	0.06	0.06	75.32	<0.0001
X_3	1	0.02	0.02	23.09	0.0007
X_1 X_2	1	0.00	0.00	0.15	0.7081
X_1 X_3	1	0.00	0.00	0.08	0.7844
X_2 X_3	1	0.00	0.00	0.02	0.8848
X_1^2	1	0.04	0.04	52.94	<0.0001
X_2^2	1	0.00	0.00	0.02	0.8957
X_3^2	1	0.19	0.19	251.14	<0.0001
Error	5	0.00	0.00	-	-
Total	19	2.27	-	-	-

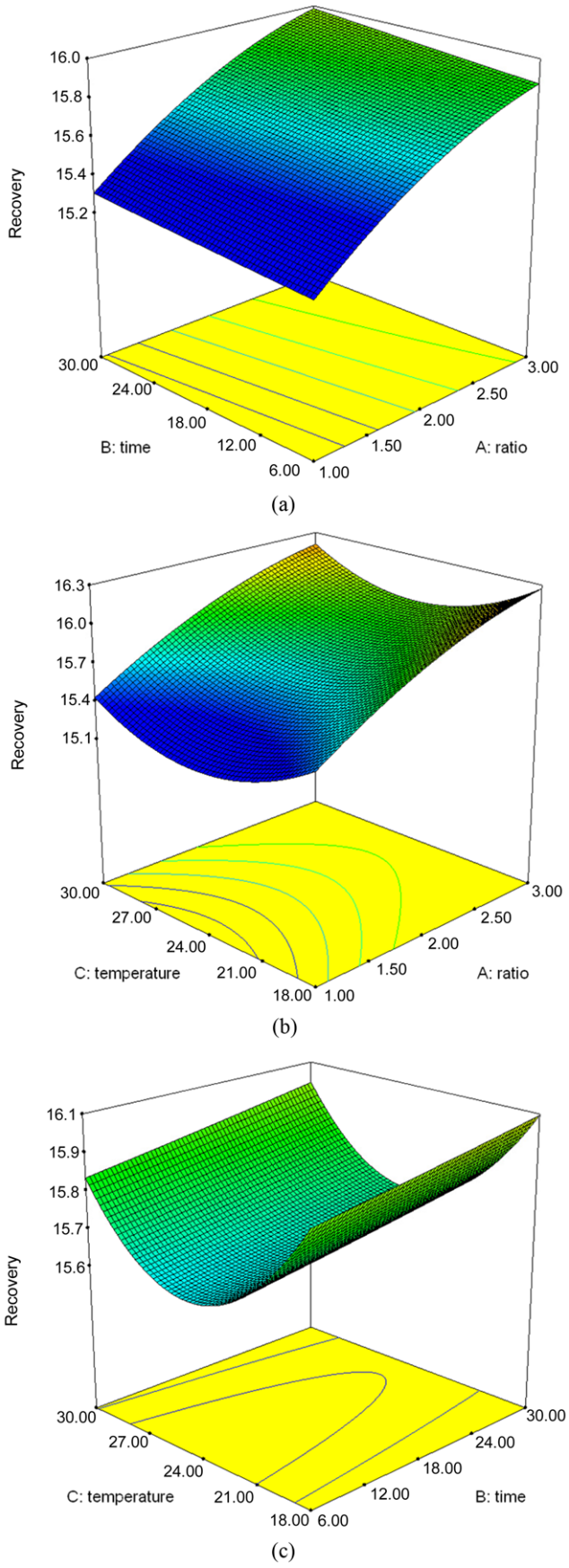

Fig. 6. Three-dimensional contour plots of three factors on recovery of pullulan by isopropanol.

order polynomial equation:

$$Y_2=15.66+0.39X_1+0.062X_2-0.033X_3+3.050\times10^{-3}X_1X_2-2.351\times10^{-3}X_1X_3+1.101\times10^{-3}X_2X_3-0.11X_1^2+2.265\times10^{-3}X_2^2+0.30X_3^2 \quad (4)$$

Where Y is the response, that is, recovered pullulan, and X_1, X_2, and X_3 are the coded values of the test variables - volume ratio of isopropanol to the supernatant, reaction time, and temperature, respectively. After the neglect of insignificant terms (on the basis of "probe>F" which are more than 0.05), the model Eq. (4) was modified to reduced fitted model Eq. (5):

$$Y_2=15.66+0.39X_1+0.062X_2-0.033X_3-0.11X_1^2+0.30X_3^2 \quad (5)$$

The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.9967. The model can explain 99.67% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9937) is also very high to advocate for the high significance of the model [20,21]. The Pred. R^2 of 0.9863 is in reasonable agreement with Adj. R^2 of 0.9937. The model *F*-value of 332.90 implied that the model was significant. There is only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. From the statistical results obtained, it was shown that the above models were adequate to predict the recovery of pullulan using isopropanol within the range of variables studied. The optimal conditions for recovery of pullulan using isopropanol were the same as those by using ethanol. The maximum recovered pullulan of 16.34 g/L was predicted by the model. The recovery yield of pullulan from the supernatant was 85.5%.

The three-dimensional response surface was generated to study the interaction among three factors tested and to visualize the combined effects of factors on the response of recovery of pullulan by isopropanol, as shown Fig. 6. The combined effects of each pair of factors also demonstrated that recovery of pullulan considerably increased with the increased volume ratio of isopropanol to supernatant, indicating that the volume ratio of isopropanol to supernatant has also a significant effect on the responses. Isopropanol gave maximum precipitation of pullulan followed by ethanol and tetrahydrofuran [25]. In current processes for production of xanthan, it is concentrated by precipitation with isopropanol [26]. The FDA regulations for preparation of good grade xanthan gum prescribe the use of isopropanol for precipitation [14]. Precipitation of pullulan from supernatant by using two volumes of ice-cold isopropanol increased with the reaction time up to 12 h and thereafter recovery yield was constant [25]. In this study, the amount of diatomite used to apply a filter press and the flow rate of the culture broth through a continuous flow centrifuge was optimized for removal of cells from the culture broth of *A. pullulans* HP-2001. And the conditions for recovery of pullulan from supernatant by ethanol or isopropanol were optimized using the response surface method.

CONCLUSION

Even though the final powder of xanthan contains 85% pure xanthan, 5% biomass, and 10% moisture, nearly 50% of the total cost for production of xanthan is attributed to recovery of xanthan in the downstream process [26]. Due to results that cells from the culture broth of *A. pullulans* HP-2001 are not completely removed using either filter press or continuous flow centrifugation alone, a sequen-

tial process of filter press followed by continuous flow centrifugation can be advised for complete removal of cells from the culture broth. By utilizing the statistical methodology, the optimal conditions for recovery of pullulan were the ratio of ethanol (or isopropanol) to supernatant of 3.0 : 1.0, reaction time of 30 h, and temperature of 18 ºC. The maximum recovered pullulans using ethanol and isopropanol were 15.24 and 16.34 g/L, respectively, which recovery yields were 79.8 and 85.5%. It is indicated that the optimized conditions for recovery of pullulan have much applied value in the industry. The results from this study will be a useful tool for removal of cells and recovery of microbial exopolysaccharides to develop a simple and cost-effective process for downstream.

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