ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Optimization of Media Components for Pullulan Production

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ABSTRACT

In this study pullulan was produced from *Aureobasidium pullulans* under submerged state fermentation. The effect of various process variables was studied for maximum pullulan production. Five medium factors, from out of fifteen medium components, were screened by Plackett-Burman design experiments and subsequent optimization process to find out the optimum values of the selected variables. Response surface methodology was used to evaluate the relationships between selected operating variables and pullulan yield. The developed regression model was fitted with R^2 = 0.99 and the results show that the regression models adequately explain the data variation and represent the actual relationships between the variable and responses. A three dimensional response surfaces were generated to study the interaction. Optimal medium were: (g/L), sucrose, 69.74; yeast extract, 4.0; K_2HPO_4, 1.806; $(NH_4)_2SO_4$, 1.789; and NaCl, 1.660. The extracted polymer was characterized by FTIR study

Keywords pullulan; placket burman design; response surface methodology; FTIR

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INTRODUCTION

Pullulan is an extra-cellular, linear, unbranched, water soluble, microbial nonionic polysaccharide. It is composed of linear α- (1$\rightarrow$6) linked maltotriose units and a small number of α- (1$\rightarrow$4) linked maltotetraose units, which endows pullulan with structural flexibility and superior solubility [1]. Efficient producers of pullulan are the yeast-like fungus *Aureobasidium pullulans*. Pullulan from *Aureobasidium pullulans* has many distinctive properties such as non-hygroscopic, nonreducing, easily water soluble, high adhesion, excellent film-forming ability, large surface area, elasticity, and biocompatibility. Owing to its distinctive 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, agricultural, and chemical industries and even in lithography [2, 3]. Despite these applications, the price of pullulan is still three times higher than that of other polysaccharides such as xanthan and dextran, which limits its large-scale utilizations. The high price of pullulan is attributed to several factors, including low product yield. However, with the limitations of low pullulan yield and raw material utilization ratio, it is important to enhance the yield of pullulan during fermentation which will make the process economically compatible [4]. During fermentation process, product concentration and yield depends on several factors and one of them being interaction among the media components. Statistic-based experimental designs have proved to be more efficient than classical one-at-a-time method, which is tedious and time-consuming, do not screen multi-variables, and do not inspect the complex interactions among different factors. Besides, statistical approaches provide a systematic and efficient plan for experimentation to achieve certain goals. These methods not only reduce the number of experiments to be carried out, but also consider the interactions of many factors which may affect the yield of the product during fermentation [5]. Plackett–Burman design will identify the important variables and allow them to be ranked in the order of importance to decide which variables are to be investigated in a more detailed study and to determine the optimum value to be used [6].

Response Surface Methodology (RSM) is one such collection of mathematical and statistical technique in which interactions between multiple process variables can be identified with fewer experimental trials. It is widely used to optimize the operational variables for experiment designing, modeling, evaluating the effects of factors and analyzing optimum conditions of factors for desirable responses. There are various advantages in using statistical methodologies, in terms of rapid and reliable short-listing of process conditions, examining the effective factors, minimizing the error in determining the effect of parameters and quantifying the relationship between one and/or more major responses, by carrying out a limited number of experiments [7]. It usually involves an experimental design such as central composite design (CCD) to fit a second-order polynomial by least-squares technique. An equation is used to relate the test variables and report the combined effect of all the test variables in the response. This design helps to estimate coefficient of quadratic model and has been extensively used for bioprocess optimization.

Earlier, researchers suggested that RSM for media optimization to enhance pullulan production using as synthetic medium containing sucrose as carbon source [8]. Some researchers applied RSM for media optimization to produce pullulan. Various researchers have optimized medium constituents and process parameters for increased pullulan yield [9]. Previously, our group has reported pullulan production by yeast-like fungus *A. pullulans* using batch kinetic study [10]. The objective of the present investigation included optimization of pullulan production from *A. pullulans* by RSM and examination of the interactions between the media components on pullulan yield. The medium components were screened by central composite design (CCD) and CCD was applied to optimize the significant factors. In order to achieve the optimum conditions, the existing study was carried out in two stages: firstly, the PlackettBurman (PB) design was applied to select the most significant components, which affect the Pullulan production. Secondly, the central composite design was employed to obtain the optimum level of the significant factors by developing a model followed by other statistical tests such as analysis of variance (ANOVA), coefficient of determination, and 3D contour for the pullulan production.

MATERIALS AND METHODS

Microorganisms and culture conditions

Fungal strain *Aureobasidium pullulans* (MTCC 2195) was obtained from Institute of Microbial Technology (IMTECH), Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. Culture was maintained on agar slant (1.0 % (w/v) glucose, 0.5 % (w/v) peptone, 0.3 % (w/v) malt extract, 0.3 % (w/v) yeast extract, and 2.0% (w/v) agar) at 4^{o}C and subculturted every three days.

Preparation of inoculum medium

The standard inoculum medium contained (g/L), sucrose, 60.0; yeast extract, 3.0; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.4; and NaCl, 1.5, and distilled water 1 L. The medium was autoclaved for 20 min at 121 °C. Prior to autoclaving, the pH of the medium was adjusted to pH = 7.0 using 1N NaOH and 1N HCl. A loopful of culture from the stock culture was inoculated to the medium. The inoculums developed in a 250-ml conical flask containing 50 ml of the medium and incubated for 48 h at 28 °C on a rotary shaker (at 250 rpm). The two-day grown culture was then used as inoculum for shake-flask cultures [11].

Screening of important media components using Plackett–Burman design

This study was done for screening medium components with respect to their effects on pullulan production using *A. pullulans*. The media components are peptone, malt extract, yeast extract, $MgSO_4.7H_2O$, $ZnCl_2$, $FeSO_4.7H$ O, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MnSO_4.H2O$, sucrose, NaCl, $NaNO_3$, KCl, and tween-80. The fifteen variables of medium components were screened at two different levels, maximum and minimum, using Plackett–Burman design. The design and levels of individual variable are shown in Table 1. The statistical software package, MINITAB version 15.0, was used to create the design and regression analysis of the experimental data (12].

Table 1: Media Components and their high and low concentrations.

Variables	Independent variables	(+) values (g/L)	(–) values (g/L)
X_1	Peptone	10	1
X_2	Malt extract	10	1
X_3	Yeast extract	5	1
X_4	$MgSO_4.7H_2O$	1	0.1
X_5	$ZnCl_2$	1	0.1
X_6	$FeSO_4/7\ H_2O$	1	0.1
X_7	KH_2PO_4	2	0.1
X_8	K_2HPO_4	2	0.1
X_9	$(NH_4)_2SO_4$	5	0.5
X_{10}	$MnSO_4\ H_2O$	0.5	0.05
X_{11}	Sucrose	50	5
X_{12}	NaCl	5	0.5
X_{13}	$NaNO_3$	1	0.1
X_{14}	KCl	5	0.1
X_{15}	Tween-80	1	0.1

Optimization of the independent variables by response surface methodology

The second step of media optimization is the screened medium components affecting pullulan production were optimized using central composite design (CCD); the experimental design and statistical analysis were performed according to the RSM using Design-Expert software (Trial Version 9, Stat-Ease, 2014). A 1/2-fraction CCD with single blocks was applied to investigate and optimize the operating variables affecting the pullulan yield. The dependent variable or response was the final pullulan yield % (g/L). The factors were examined at five different levels (relatively low, low, basal, high, and relatively high) coded (-2, -1, 0, +1, and + 2). A 2^k (k=5) factorial central composite experimental design with six replicates at the center point resulted in 32 experiments which covers the entire range of spectrum of combinations of variables that were used to optimize the screened media components for pullulan yield. The relationship between the operating variables and the response is described in the following empirical quadratic polynomial equation

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad .....(1)$$

Where Y is the response variable (pullulan yield), β_0 is the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, and β_{ij} the interaction coefficients; X_i and X_j represent the coded independent variables. The regression coefficients of individual linear, quadratic, and interaction were determined by the software, and the significance of all terms was assessed statistically using F-value at a probability (P) of 0.001, 0.01, or 0.05. They were further used to make statistical calculations to generate contour maps with the help of the regression model. The three-dimensional (3D) plots were generated by keeping three variables constant at the center point and varying the other two variables within the experimental range [13].

Estimation of pullulan

Batch fermentation

Batch fermentations were carried out according to experimental design. All the experiments were carried out in 250-mL Erlenmeyer flasks. 50 ml of the sterilized fermentation medium was cooled and inoculated with 5% (v/v) inoculum of two-day pregrown culture. The flask was kept in an incubator–shaker for 168 h at 28°C and at 200 rpm. The sample was withdrawn at a regular time interval and assayed for biomass, pullulan, and residual sugar content. All the runs were carried out in triplicates, and the average was tabulated.

Estimation of cell mass and pullulan

At specific time intervals, the flasks were removed from the incubator–shaker and the fermentation broth was analyzed for cell mass and pullulan. Dry weight of total cell mass was determined by centrifuging the fermentation broth at 15000 rpm for 20 minutes. The collected cell mass was washed twice with saline and distilled water and dried at 50°C till the mass reaches a constant weight. After the removal of cell mass, ethanol was added twice the volume of supernatant for precipitation of polysaccharide and maintained at 4°C for 1h. The precipitate was filtered through a pre-weighed No-40 Whatman filter paper and dried at 50°C overnight. The results were expressed in gram per liter [10, 11].

RESULT AND DISCUSSION

In the present investigation, the significance of 15 different media components on production of pullulan was screened in order to improve the composition of the medium. Table 2 gives the experimental plan along with the results. It was observed that the pullulan production varied between 4–25 g/L. This indicates that the selected nutritional compounds show a significant effect on production of the pullulan. Table 3 represents the results of Plackett–Burman experiment with respect to pullulan production, the effect, t (xi), standard error, p level, and confidence level of each component. The variables were screened at the confidence level of 99.5% on the basis of their effects. If the media components showed significance at or above 99.5% confidence level and its effect was negative, it stipulated that the component was effective in pullulan production, but the amount required was lower than the indicated low value (–) concentration. If the effect was positive, a higher concentration then indicates high value (+) concentration was required during further optimization studies. Peptone, malt extract, and yeast extract were selected as source of nitrogen, and their confidence levels were 93.2, 99.1, and 100% at 1.0 g/L concentration. Moreover, a considerable amount of exorbitant complex nitrogen source, yeast extract, must be added to the medium to produce pullulan. Among the phosphate sources used, only K_2HPO_4 was significant at 100% and there was considerable difference in pullulan production between the two phosphate sources used. Highest production was achieved with K_2HPO_4, and thus it was selected for further studies. From the sulfate sources used, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ found to be 98% significant for pullulan production. Only $(NH_4)_2SO_4$ was significant at 99.9% and had a positive effect on pullulan production. It reveals that higher concentration it is, needed for further optimization. The confidence level of the chloride salts which are used like ZnCl having 41.7% significance, KCl having 95.5% significance. Only NaCl was found to be the significant factor among the chloride salts due to the higher confidence level (100%). Other media components such as sodium nitrate and tween-80 were found to be insignificant. Sucrose which is used as carbon source for pullulan production was significant at 100% level.

The confidence level of components peptone, malt extract, $MgSO_4.7H_2O$, ZnCl, $FeSO_4.7H_2O$, KH_2PO_4, $MnSO_4.H_2O$, $NaNO_3$, KCl, and tween-80 were below 99.5% in pullulan production. The rest of the components, sucrose, yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, and sodium chloride, showed confidence level at or above 99.5%. Table 4 shows the five independent variables (sucrose, yeast extract, K_2HPO_4, $(NH_4)_2SO_4$, and NaCl) and their concentrations at different coded and actual levels of the variables employed in the design matrix. Central composite design matrix of the independent variables in coded units (experimental design) along with experimental values of response is given in Table 5. Based on CCD experiment, the effects of five independent variables including sucrose, yeast extract, K_2HPO_4, $(NH_4)_2SO_4$, and NaCl on pullulan production are shown in Table 6. The predicted and observed responses were reported. As shown in Fig. 1, the predicted data is plotted as a function of experimental data on a 45-degree line; the even distribution of these data points below and above the 45-degree line represents a good agreement between the predicted and experimental results. The effects of sucrose (X_1), yeast extract (X_2), $(NH_4)_2SO_4$ (X_3), K_2HPO_4 (X_4), and NaCl (X_5) on pullulan yield were statistically significant ($P < 0.05$). Among the interaction combinations, four combinations X_1X_2, X_1X_4, X_2X_3,X_3X_4, and X_3X_5 were found to significantly ($P < 0.05$) affect the pullulan yield, whereas X_1X_3, X_1X_5, X_2X_4, X_2X_5, and X_4X_5 have no significant effect ($P > 0.05$) on pullulan yield. In addition, the three quadratic terms X_1^2 , X_3^2 , and X_4^2 have significant effects ($P < 0.05$) on pullulan yield, whereas X_2^2 and X_5^2 have no significant effect due to high probability value($P > 0.05$). After using the designed experimental data and eliminating some terms, the polynomial model for pullulan produced (Y) was regressed by considering only the significant terms ($P < 0.05$) as shown in the following equation:

$$Y = 25.20 + 3.80.X_1 - 0.32.X_2 + 1.22.X_3 + 1.24.X_4 – 0.40.X_5 - 2.63.(X_1)^2 – 1.36.(X_3)^2 – 1.11.(X_4)^2 + 0.38.(X_1X_2) + 0.76.(X_1X_4) - 0.57.(X_2X_3) + 0.660.(X_3X_4) + 0.42. X_3X_5$$

The regression model was further evaluated using analysis of variance (ANOVA), and the results are shown in Table7. The low probability value demonstrates that the model is highly significant. The goodness of fit of the model was further checked using the determination coefficient (R^2). The value of R^2 closer to 1 show that there is a good correlation between the predicted and experimental values [14]. Hence, the value of R^2 (0.9933) illustrates a good relationship between the predicted and experimental values. Furthermore, the value of R^2 also indicates that the model can explain 99 %of the total variation for the pullulan yield to the operating parameters. To visualize the relation between the operating variables and pullulan yield, response surface plots and contour maps were generated and presented using Design-Expert (version 9.0) based on the regression model.

The 3D surface plots and contour maps showing the pullulan yield are presented in Fig. 2a–c. In the 3D surface plots and contour maps, the pullulan yield was calculated using Eq. (2) along with two continuous variables, while the other three variables were fixed as constant at central level. Figure 2a shows the effects of sucrose and $(NH_4)_2SO_4$on pullulan yield at the fixed level of yeast extract, K_2HPO_4, and NaCl. The two variables were observed to have an obvious effect on pullulan yield. Concentrations near mid-points were observed as optimal values for sucrose and $(NH_4)_2SO_4$. The 3D surface plot and the contour map based on two variables, sucrose and K_2HPO_4, are shown in Fig. 2b, whereas the other three variables were kept at a centre level. It can be seen that the two variables had a positive impact on pullulan yield. Simultaneously increasing the two variables caused a linear increase in the pullulan yield. However, a decrease in the pullulan yield was observed with further increases in the two variables. The maximum value of pullulan yield was 27.76 g/L when sucrose and K_2HPO_4 were approximately 70 g/L and 2.0 g/L, respectively. The surface plot and the contour map in Fig. 2c show the effects of $(NH_4)_2SO_4$ and K_2HPO_4 on pullulan yield when the other three variables were fixed at centre level. The two variables were observed to have an obvious effect on pullulan yield. At the lowest level of $(NH_4)_2SO_4$ or K_2HPO_4, the pullulan yield was found to increase slightly at first and then decrease with continuously increasing $(NH_4)_2SO_4$ and K_2HPO_4. A higher pullulan yield can be obtained at higher levels of both the variables, i.e., the pullulan yield was increased with simultaneously increasing of the values of $(NH_4)_2SO_4$ and K_2HPO_4. The maximum value of pullulan yield of 28.2 g/L was achieved when the values of $(NH_4)_2SO_4$ and K_2HPO_4 were increased to approximately 1.7 g/L and 1.8g/L, respectively.

For selection of the optimum conditions and range, the model was analyzed separately. From the validation report, the required criteria were selected with maximum pullulan as the target. Choice of solutions was automatically retrieved by the software. The target goal was to reach maximum pullulan yield with limited yeast extract supplementation as yeast extract is one of the costly nutrient sources for fermentation. So, minimum yeast extract was selected. The level of sucrose was selected in range from 50 to 70 g/L. The K_2HPO_4, $(NH_4)_2SO_4$ and NaCL were also in the range from 1.0 to 2 g/L. The maximum pullulan yield 28.035 g/L was obtained at optimal level were sucrose, 69.74; yeast extract, 4.0; K_2HPO_4, 1.806; $(NH_4)_2SO_4$, 1.789; and NaCl, 1.660. Pullulan production using *A. pullulans* may increase further if the fermentation conditions are optimized in the future. In other reports, maximum pullulan production was 23 g/L [15, 16] and 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. The optimum concentration of NaCl noticed here for *A. pullulans* is lower than that is generally used in other studies [17]. In addition, disturbing the metabolism of *A. pullulans* by regulating the osmotic pressure, ionic strength, and Na+/K+- ATPase, K_2HPO_4 provides phosphorous for *A. pullulans*. Thus, the optimal concentration of K_2HPO_4 used is also lower than that is generally used in other studies [18]. Yeast extract influence the metabolism of *A. pullulans* and the optimum concentration of yeast extract, 4 (g/L) used is in agreement with the other studies. In addition to the yeast extract, $(NH_4)_2SO_4$ can also influence the metabolism of *A. pullulans* by providing nitrogen for the microorganism, and the optimum concentration of $(NH_4)_2SO_4$, 1.789 used is in a lower with that reported in other study using 5 (g/L) of $(NH_4)_2SO_4$ [19].

Table 2: PB Design Along with Observed and Predicted Pullulan yield

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Pullulan (g/L)	
																Obs.	Pred.
1	+	+	−	+	−	+	−	−	−	−	+	−	−	+	−	12.5	14.449
2	−	+	−	+	−	−	−	−	+	−	−	+	−	+	+	8.6	6.895
3	+	+	−	−	+	+	+	+	+	−	−	−	+	+	−	2.2	0.103
4	−	+	−	+	+	−	−	+	+	+	+	+	−	−	−	24.6	23.629
5	−	−	−	−	+	−	−	+	−	+	+	−	−	+	+	20.6	21.047
6	−	+	−	−	−	−	+	−	−	+	−	+	+	−	−	8.5	8.135
7	+	−	−	−	+	+	−	+	+	+	−	+	−	+	−	6.4	7.709
8	−	−	+	+	−	+	+	+	−	+	−	+	−	−	−	20.5	20.729
9	−	+	+	+	−	+	−	+	−	−	−	−	+	−	−	12.98	13.371
10	−	−	+	−	+	+	−	−	+	+	+	+	+	−	−	24.02	22.011
11	+	+	+	+	+	−	−	−	+	+	−	+	+	+	−	7.08	9.255
12	+	−	+	+	+	−	+	−	+	−	−	−	−	+	−	10	9.009
13	−	−	−	+	+	−	+	+	+	−	+	−	+	−	−	16.4	20.175
14	+	+	+	−	+	−	+	−	−	−	−	+	−	−	+	12.04	12.863
15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	8	8.137
16	+	−	+	−	−	−	−	+	−	−	+	−	+	+	−	26.1	24.127
17	−	−	+	+	+	+	+	−	−	−	+	+	−	+	+	24.6	26.079
18	−	−	+	−	−	+	−	+	+	−	−	+	+	+	+	11	12.769
19	−	+	+	+	+	+	−	−	−	+	+	−	+	+	+	19.9	18.789
20	+	+	−	−	−	+	+	−	+	+	+	−	+	−	+	7.08	9.361
21	+	−	+	+	−	−	+	+	+	+	+	−	−	−	+	27.08	25.759
22	+	−	−	−	−	+	−	−	+	−	+	+	−	−	+	17.4	16.899
23	+	−	+	−	+	−	−	−	−	+	−	−	+	−	+	8.9	9.725
24	+	−	−	+	−	+	+	−	−	+	+	+	+	+	−	19.5	18.599
25	+	−	−	+	+	+	+	+	−	−	−	+	+	−	+	13	11.621
26	−	+	−	−	+	−	+	+	−	−	+	+	+	+	+	21.4	20.621
27	−	+	+	−	−	+	+	+	+	+	−	−	−	+	+	8	8.439
28	−	+	+	−	+	+	+	−	+	−	+	−	−	−	−	18.8	17.613
29	+	+	+	+	−	−	−	+	+	−	+	+	+	−	+	26	25.937

30	+	+	+	+	−	−	−	+	+	−	+	+	+	−	+	26	25.539
31	+	+	−	+	+	+	−	+	−	+	−	−	−	−	+	8.2	7.539
32	−	−	−	+	−	−	+	−	+	+	−	−	+	+	+	4.4	3.509

Table 3: Effects and anova for selected variables

Terms	Effect	Coeffi.	Std. dev. Coeffi.	t	P	Confidence (%)
Intercept		15.081	0.3517	59.94	0.000	100
1	-1.376	-0.688	0.3517	-1.96	0.068	93.2
2	-2.076	-1.038	0.3517	-2.95	0.009	99.1
3	5.314	2.657	0.3517	7.55	0.000	100
4	1.756	0.878	0.3517	2.50	0.024	97.6
5	-0.394	-0.197	0.3517	-0.56	0.583	41.7
6	-1.901	-0.951	0.3517	-2.70	0.016	98.4
7	-0.124	-0.062	0.3517	-0.18	0.863	13.7
8	3.746	1.873	0.3517	5.33	0.000	100
9	-2.779	-1.389	0.3517	-3.95	0.001	99.9
10	0.034	0.017	0.3517	0.05	0.962	3.8
11	11.436	5.718	0.3517	16.26	0.000	100
12	3.769	1.884	0.3517	5.36	0.000	100
13	-1.604	-0.802	0.3517	-2.28	0.037	96.3
14	-1.526	-0.763	0.3517	-2.17	0.045	95.5
15	-0.386	-0.193	0.3517	-0.55	0.591	40.9

Table 4. Codes and actual levels of the independent variables for design of experiment

Independent variables	Symbols	Coded levels				
		-2	-1	0	+1	+2
Sucrose, (g/L)	X_1	40	50	60	70	80
Yeast extract, (g/L)	X_2	2	4	6	8	10
$(NH_4)_2SO_4$, (g/L)	X_3	0.5	1.0	1.5	2.0	2.5
K_2HPO_4, (g/L)	X_4	0.5	1.0	1.5	2.0	2.5
NaCl, (g/L)	X_5	0.5	1.0	1.5	2.0	2.5

Table 5: Experimental design, observed yields in CCD experiments in term of pullulan yield

Run No.	Coded and real variables					Pullulan concentration (g/L)	
	X_1	X_2	X_3	X_4	X_5	Experimental	Predicted
1	-2	0	0	0	0	7.0	7.05
2	1	-1	-1	-1	-1	21.9	21.34
3	0	0	0	0	0	25.2	25.19
4	1	1	-1	1	-1	24.9	24.01
5	1	1	1	-1	-1	21.5	22.47
6	1	1	1	1	1	28.0	27.76
7	-1	1	-1	1	1	14.0	14.89
8	1	-1	-1	1	1	22.0	24.01
9	0	-2	0	0	0	26.7	25.19
10	0	0	0	0	0	25.2	25.19
11	0	0	0	0	0	25.2	25.19
12	1	-1	1	1	-1	28.2	27.76
13	0	0	0	0	-2	26.0	25.19
14	0	0	0	0	0	25.2	25.19
15	0	0	2	0	0	22.2	22.20
16	0	2	0	0	0	25.5	25.19
17	0	0	0	0	2	24.2	25.19
18	1	1	-1	-1	1	22.2	21.34
19	0	0	0	2	0	24.5	23.23
20	0	0	0	-2	0	17.0	18.28
21	-1	-1	1	1	1	18.7	18.64
22	0	0	0	0	0	25.2	25.19
23	-1	-1	-1	1	-1	16.5	14.89
24	-1	-1	-1	-1	1	15.0	15.24
25	-1	-1	1	-1	-1	18.7	16.37
26	-1	1	-1	-1	-1	16.2	15.24
27	-1	1	1	-1	1	15.5	16.37
28	0	0	-2	0	0	17.3	17.31
29	1	-1	1	-1	1	24.1	22.47
30	0	0	0	0	0	25.2	25.19
31	-1	1	1	1	-1	17.5	18.64
32	2	0	0	0	0	22.3	22.26

Table 6. Regression coefficients and significances obtained by the response surface model

Term constant	Regression coefficient	Std. deviation	P-value
Intercept	25.20	0.27	<0.0001
X_1	3.80	0.14	<0.0001
X_2	-0.32	0.14	0.0385
X_3	1.22	0.14	<0.0001
X_4	1.24	0.14	<0.0001
X_5	-0.40	0.14	0.0145
$X_1 X_1$	-2.63	0.12	<0.001
$X_2 X_2$	0.23	0.12	0.0915
X_3X_3	-1.36	0.12	<0.0001
X_4X_4	-1.11	0.12	<0.0001
X_5X_5	-0.022	0.12	0.8644
X_1X_2	0.38	0.17	0.0436
X_1X_3	0.13	0.17	0.4492
X_1X_4	0.76	0.17	0.0009
X_1X_5	0.34	0.17	0.0644
X_2X_3	-0.57	0.17	0.0059

X_2X_4	0.21	0.17	0.2433
X_2X_5	0.32	0.17	0.0832
X_3X_4	0.66	0.17	0.0024
X_3X_5	0.42	0.17	0.0293
X_4X_5	-0.18	0.17	0.3017

Table 7: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for pullulan production

Sources of variation	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	F=value	P-value
Regression	731.68	20	36.58	81.72	<0.001
Linear	426.08	5	85.22	90.36	<0.001
Square	274.25	5	54.85	22.53	<0.001
Interaction	31.35	10	3.13	7.00	0.002
Residual Error	4.92	11	0.45	-	-
Lack-of-Fit	4.92	6	0.82	-	-
Pure Error	0.000	5	0.0000	-	-
Total	736.6	31	-	-	-

R2 = 0.9933; SS, sum of squares; DF, degrees of freedom; MS, mean square; Adj R2 = 0.9812.

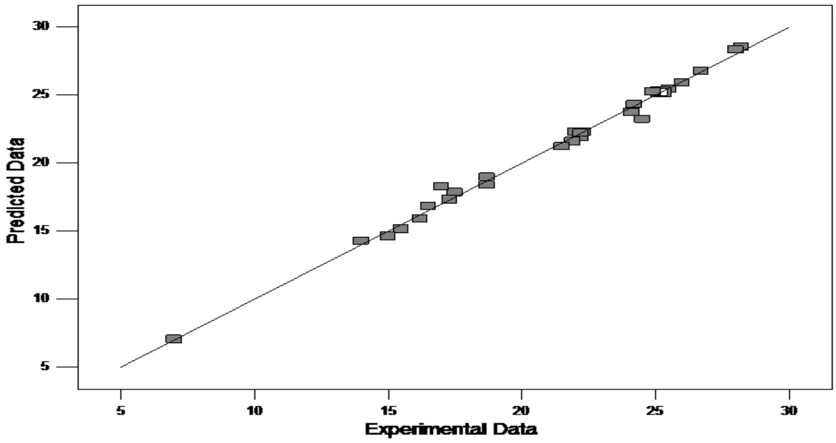

Fig.1 Predicted yield Vs Experimental Yeild

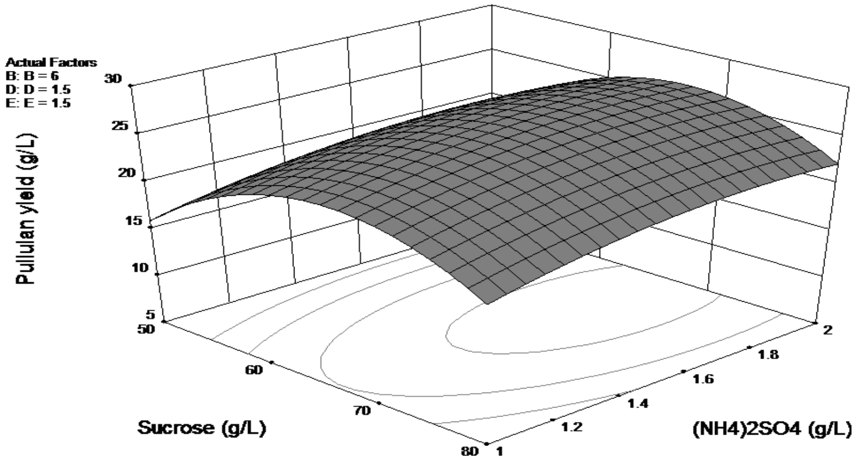

Fig.2a. Contour plots between Sucrose and $(NH_4)_2SO_4$ for the response

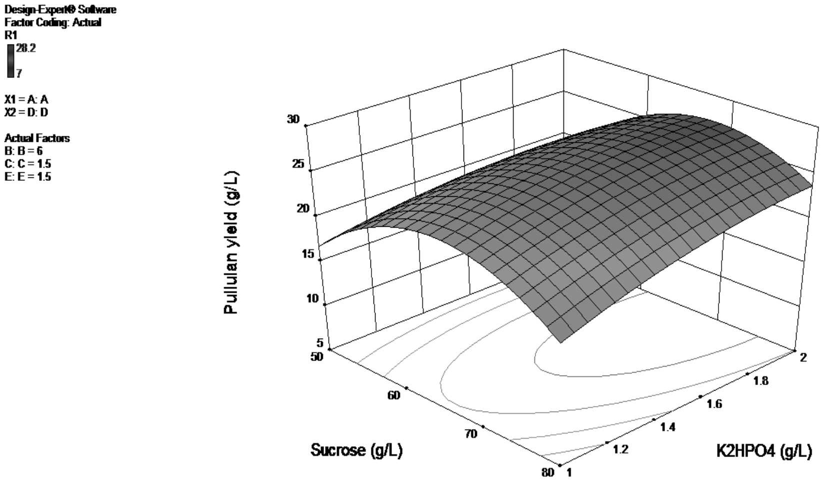

Fig.2b. Contour plots between K_2HPO_4 and sucrose for the response

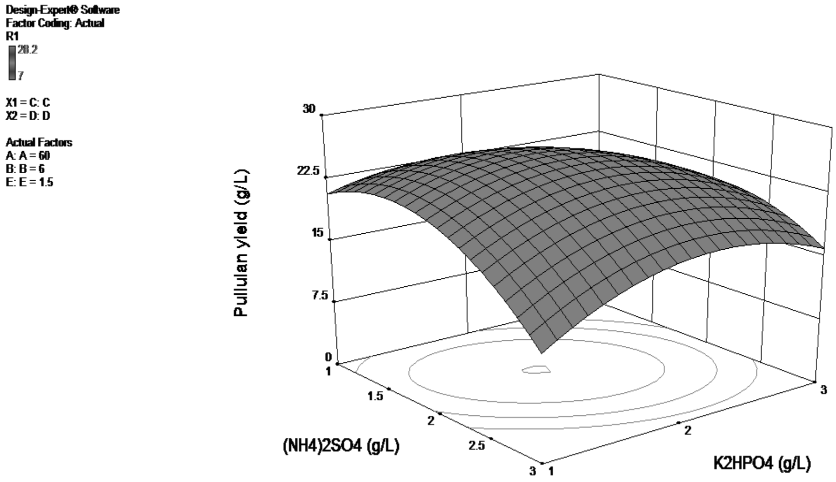

Fig.2c. Contour plots between K_2HPO_4 and $(NH_4)_2SO_4$ for the response

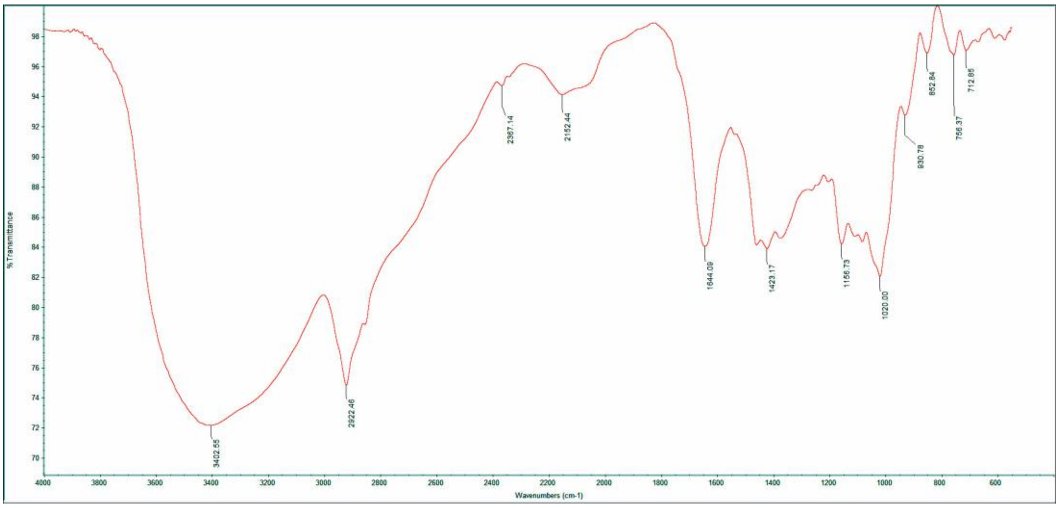

Fig.3a. FTIR Spectra for Pullulan

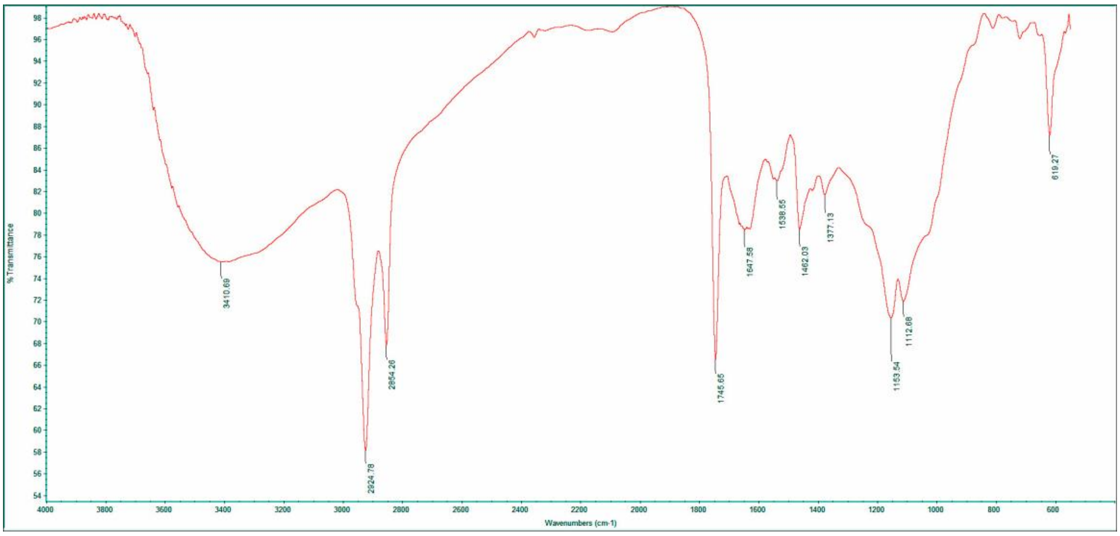

Fig.3b. FTIR Spectra for Commercial Pullulan

Characterization of the exopolysaccharide

FTIR spectra of the exopolysaccharide prepared in this study and the pullulan obtained from Sigmachemical laboratory are identical, indicating that this exopolysaccharide is primarily composed of pullulan (Fig. 3a,3b).

The strong absorption at 3402 cm^{-1} indicated that the pullulan had some repeating units of –OH as in sugars. The other strong absorption at 2922 cm^{-1} indicated a sp^3 C-H bond of alkane compounds existed in all the samples. In the specific areas (1640 – 780 cm^{-1}), which is characteristic for the pullulan molecule (Fig. 3a). The spectra of commercial pullulans as well as those for evaluated samples exhibited similar features (Fig. 3b) and thus confirming the pullulan structure of this exopolysaccharide.

CONCLUSION

In the optimization of media components for pullulan production by *A. pullulans* , the combination of Plackett-Burman design with central composite design is effective and reliable in selecting the statistically significant factors and finding the optimal concentration of those components in fermentation medium. The study demonstrates the use of a central composite design by determining the conditions leading to the optimum yield of pullulan production.The existence of interactions between the independent variables with the responses was observed. The optimal media components are as follows (g/L) : sucrose, 69.74; yeast extract, 4.0; K_2HPO_4, 1.806; $(NH_4)_2SO_4$, 1.789; and NaCl, 1.660. 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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