Understanding response surface optimization of medium composition for pullulan production from de-oiled rice bran by *Aureobasidium pullulans*

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Abstract Central composite rotatable design of RSM was used for the optimization of medium composition for pullulan production from de-oiled rice bran by *Aureobasidium pullulans* in shake-flask fermentations. The sugars from de-oiled rice bran were extracted in distilled water under moist steam pressure and the obtained de-oiled rice bran extract (DRBE) was used for the optimization of medium composition. RSM optimized medium components (DRBE sugars, 3.88%; yeast extract, 0.24%; (NH₄)₂SO₄, 0.06%; K₂HPO₄, 0.57% (w/v), and pH, 5.22) supported 5.48% (w/v) pullulan production and 0.88 (A₆₀₀/100) biomass yield. Coefficient of determination for pullulan production (0.99) and biomass yield (0.99) was close to 1.0 which justifies significance of model. Lack of fit for both responses was non-significant, which shows fitness of quadratic model. FTIR and NMR spectral attributes confirmed the structure of pullulan. XRD patterns verified the amorphous nature of pullulan. De-oiled rice bran was found as a potential substrate for pullulan production.

Keywords *Aureobasidium pullulans* · De-oiled rice bran · Pullulan · Shake-flask fermentations

Introduction

Pullulan is a linear glucan polymer mainly composed of maltotriose repeating units which are interlinked by α -(1, 6) glycosidic bond. It is a white odorless, tasteless and edible powder easily soluble in water. It is non-ionic and non-hygroscopic without any toxicity or carcinogenicity. Owing to these unique properties, pullulan and its derivatives have potential applications in biomedical (Singh and Saini, 2012, 2014; Singh et al., 2015, 2016, 2017, 2018) and food industries (Singh et al., 2008; Singh and Kaur, 2015). Pullulan is commercially produced by yeast-like fungus *Aureobasidium pullulans* in the form of amorphous slime extracellularly. The accumulation of pullulan on outer surface of the cells is due to the formation of glycolipids in fungal mycelial cells (Ono et al., 1977). Besides *A. pullulans*, a number of other microbial sources like *Tremella mesenterica*, *Cytaria hariatii*, *C. darwinii*, *Cryphonectria parasitica*, *Teloschistes flavicans*, *Rhodotorula bacarum* and *Cryphonectria parasitica* have been reported for pullulan production (Singh et al., 2008). Recently, *Rhodospiridium paludigenum* has been reported as potent pullulan producer (Singh and Kaur, 2018).

Pullulan production involves fermentation of a medium supplemented with carbon, nitrogen and other essential nutrients. The commercial production of pullulan is very expensive which limits its use in food and pharmaceutical industries. The pullulan production can also be carried out by using cheap substrates. The waste generated by agro-based industries is rich in essential nutrients and it can be used as a substrate for pullulan production. The waste from various agro-industries like beet molasses (Goksungur et al., 2004), sugarcane molasses (Srikanth et al., 2014), corn steep liquor and de-oiled jatropha seed cake (Mehta

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et al., 2014), etc. have been used for pullulan production at laboratory scale.

The rice milling industries generate a number of by-products i.e. rice bran, rice husk, etc. Rice bran contains highly valuable oil and the extraction of oil from it generates de-oiled rice bran. De-oiled rice bran is a rich source of carbohydrates (58.5%) i.e. cellulose (16.0%), hemicellulose (29.8%), and lignin (12.7%). Apart from them, it also contains proteins (16.9%), total fiber (9.6%), and fat (1.5%) (Sunphorka et al., 2012). Owing to these properties, de-oiled rice bran can be explored as a substrate for pullulan production. In the present investigation, de-oiled rice bran extract was used for formulation of medium for pullulan production by *A. pullulans* in shake-flask fermentations. The response surface optimization of medium composition using de-oiled rice bran has significantly enhanced the pullulan production as compared to sucrose based medium as reported earlier (Singh et al., 2009b). This is the first report on the production of pullulan from de-oiled rice bran on a laboratory scale.

Materials and methods

Microorganism and inoculum preparation

Aureobasidium pullulans MTCC 6994, an isolate of our laboratory (Singh and Saini, 2008a) was used for present investigation. It was maintained on potato dextrose agar slopes as described previously (Singh et al., 2009b). Inoculum was prepared in a medium containing (%): sucrose 5.0, K_2HPO_4 0.2, $(NH_4)_2SO_4$ 0.1, NaCl 0.05, $MgSO_4 \cdot 7H_2O$ 0.005, $FeSO_4$ 0.001, $MnSO_4$ 0.001, $ZnSO_4$ 0.001 as reported earlier (Singh et al., 2009b).

Preparation of de-oiled rice bran extract

De-oiled rice bran was collected from Lakshmi Energy and Food Ltd., Khamano, Punjab, India. De-oiled rice bran (5–35%, w/v) was added in distilled water in Erlenmeyer's flasks fitted with Teflon corks and kept under pressure at 121 °C for 15 min in an autoclave. Then, resultant mixture was cooled to room temperature. Thereafter, it was filtered using Whatmann filter paper no. 1. The filtrate obtained was analyzed for total sugars, reducing sugars and used for pullulan production.

Experimental design for pullulan production from de-oiled rice bran and statistical analysis

Central composite rotatable design (CCRD) of response surface methodology (RSM) was used to optimize the pullulan production from de-oiled rice bran extract

(DRBE) in shake-flask fermentations. The statistical software Design Expert version 10.0.3 (Stat-Ease Inc., Minneapolis, MN, USA) was used to determine the optimal combination of variables and to study the response patterns. The independent variables investigated for optimization are: DRBE sugars (0.77–6.23%, w/v), yeast extract (0.02–0.38%, w/v), $(NH_4)_2SO_4$ (0.04–0.14%, w/v), K_2HPO_4 (0.04–0.76%, w/v), and pH (4.18–7.82). During shake-flask fermentations, $MgSO_4 \cdot 7H_2O$ (0.04%, w/v) and NaCl (0.20%, w/v) were kept constant in the fermentation medium. Each variable was studied at five coded levels, i.e., -1.821 , -1.000 , 0.000 , 1.000 , and 1.821 for two responses viz. pullulan production (% w/v) and biomass yield ($A_{600}/100$) (Table 1). A total 26 combinations were performed, with 10 axial points, 11 factorial points and 5 replicate centre points. The experimental runs at the centre points (1, 3, 11, 17 and 22) were used to determine the inaccuracy in the experiments. For each experimental run, production medium (100 mL) in Erlenmeyer flask (250 mL) was inoculated with 5% (w/v) inoculum and kept at 30 °C, under agitation (150 rpm) for 7 days. After 7 days of fermentation, each flask was analyzed for pullulan production and biomass yield. The following second-order polynomial equation was employed to analyze the experimental data:

$$Y = A_0 + \sum A_1X_1 + \sum A_{11}X_1^2 + \sum A_{12}X_1X_2 + E \quad (1)$$

where Y is the response, A_0 is constant coefficient, A_1 , A_{11} and A_{12} are regression coefficients of the model, while X_1 and X_2 represents independent variables and E is error of the model. Besides, analysis of variance was also employed to analyze the significance of designed model on the basis of p value ($p < 0.05$) and lack of fit ($p > 0.05$). Coefficient of determination (R^2) was used to determine the quality of fitted regression model terms. Furthermore, the optimal levels of variables obtained after statistical analysis of data were also scrutinized using 3D surface graph plots using Design Expert program (10.0.3 version, Stat-Ease Inc., Minneapolis, MN, USA).

Validation of experimental model

The validation of designed model was performed by experimental trials of 3 runs of different combinations of five factors, generated by the software. The comparison between observed and predicted values of only the experimental run supporting the maximum pullulan production and biomass yield was used to testify the authenticity of the model.

Table 1 Values of coded levels used for the experimental design

Variables and responses	Symbol	Units	Actual levels of coded factors				
			− 1.821	− 1.000	0	1.000	1.821
De-oiled rice bran extract sugars ^a	A	%	0.77	2.00	3.50	5.00	6.23
Yeast extract	B	%	0.02	0.10	0.20	0.30	0.38
(NH ₄) ₂ SO ₄	C	%	0.04	0.06	0.09	0.12	0.14
K ₂ HPO ₄	D	%	0.04	0.20	0.40	0.60	0.76
pH	E		4.18	5.00	6.00	7.00	7.82
Pullulan production	R1	%					
Biomass yield	R2	OD (A ₆₀₀ /100)					

^aDe-oiled rice bran in distilled water was kept at 121 °C for 15 min. Extract was separated by filtration and analyzed for total sugars

Analytical techniques

Extraction of pullulan

After 7 days of shake-flask fermentations, the culture broth was centrifuged (5000 rpm, 4 °C, 10 min) and cell-free extract was precipitated with two volumes of chilled isopropyl alcohol for pullulan extraction as described previously (Singh et al., 2009a).

Estimation of total sugars

De-oiled rice bran extract was investigated for total sugars by anthrone method (Scott and Melvin, 1953).

Estimation of reducing sugars

The reducing sugars from the de-oiled rice bran extract were estimated by DNSA method (Miller, 1959).

Biomass yield

The fermentation broth was diluted 100 times with cell-free broth and its absorbance was taken at 600 nm using a spectrophotometer (DR/2500, HACH, USA) against cell-free broth as blank. Biomass yield was expressed as A₆₀₀/100.

Characterization of pullulan

Structural analysis of crude and pure pullulan (Sigma-Aldrich Company, USA) was carried out by Fourier-transform infrared (FTIR) spectroscopy using potassium bromide technique (Perkin-Elmer, Norwalk, CT). The structural confirmation of crude and pure pullulan was also carried out by ¹³C and ¹H nuclear magnetic resonance (NMR) spectroscopy on a Bruker AVANCE II 400 NMR spectrometer with a narrow bore magnet and 5 mm 1H/BB

(109Ag-31P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.) at 296 K. Differential scanning calorimetry (DSC) of crude and pure pullulan was carried out using a thermal analysis calorimeter (EVO 131, SETARAM instrumentation, France). Both exothermic and endothermic peaks were observed. X-ray patterns of crude and pure pullulan were scanned and analyzed using an X-ray diffractometer (XPert-Pro, Poland) with CuK α radiation ($\lambda = 0.15406$ nm) at a voltage of 45 kV, 40 mA and $2\theta = 5$ – 99° with a scanning speed of 4° min^{-1} . Further to study morphology, crude pullulan powder was mounted onto the sample holder, coated with gold particles in presence of argon gas and photographed using a scanning electron microscope (JSM-6610LV, Jeol, Tokyo, Japan) at 15 kV accelerating voltage, 1–10 μm working distance, and a probe current of 3×10^{-11} A.

Statistical analysis

All the results are an average of three independent experiments. Data has been represented as $\pm$ standard error mean.

Results and discussion

Analysis of de-oiled rice bran extract

An increase in sugar concentration was observed with the increase in concentration of de-oiled rice bran during extraction under pressure in an autoclave. The total sugar content in the extract from 5, 10, 15, 20, 25, 30 and 35% (w/v) de-oiled rice bran was 0.80, 1.66, 2.10, 3.50, 4.57, 5.05 and 6.18%, respectively. The corresponding reducing sugars were 0.041, 0.053, 0.069, 0.076, 0.084, 0.095 and 0.099% in de-oiled rice bran extract. The de-oiled rice bran extract obtained were used to adjust the sugar

concentration during response surface optimization for pullulan production by *A. pullulans* in shake-flask fermentations.

Experimental design and statistical analysis

A significant interaction was observed between media components for pullulan production and biomass yield during response surface optimization (Table 2). The evaluation of present design indicated that quadratic model is the most suitable one for this set of experiments. The p -value and F -value were used to statistically evaluate the overall significance of regression model, fitness of the model terms and to evaluate the specific regression terms. The smaller p -value and greater F -value shows the statistical significance of generated model (Mehta et al., 2014; Singh et al., 2009b). The p -value less than 0.05 verified the

significance of present model terms. The higher F -value for pullulan production (28.43) and biomass yield (37.38) validated the significance of generated model (Table 3). The Fisher's statistical test for analysis of variance (ANOVA) confirmed the statistical significance and reliability of regression model (Table 3). The ANOVA suggested validation of the model terms for development of quadratic equation and the final equation in terms of coded factor for pullulan production and biomass yield are:

$$\begin{aligned} \text{Pullulan production} = & +5.58 + 0.3322A - 0.5711E \\ & - 0.5135BD - 0.8221A^2 \\ & - 0.7694E^2 \end{aligned} \quad (2)$$

Table 2 CCRD matrix with observed and predicted results for pullulan production and biomass yield

Run	A	B	C	D	E	Pullulan production (%)			Biomass yield ($A_{600}/100$)		
						Observed value	Predicted value	Standard deviation	Observed value	Predicted value	Standard deviation
1	3.50	0.20	0.09	0.40	6.00	5.80	5.72	0.056	0.830	0.862	0.0226
2	2.00	0.10	0.06	0.20	5.00	3.36	3.52	0.113	0.587	0.590	0.0021
3	3.50	0.20	0.09	0.40	6.00	5.62	5.72	0.070	0.850	0.862	0.0084
4	3.50	0.20	0.09	0.40	7.82	2.00	1.98	0.014	0.580	0.581	0.0007
5	5.00	0.10	0.12	0.60	5.00	4.72	4.80	0.056	0.738	0.739	0.0007
6	6.23	0.20	0.09	0.40	6.00	3.47	3.33	0.098	0.620	0.616	0.0028
7	3.50	0.20	0.04	0.40	6.00	5.92	5.78	0.098	0.889	0.885	0.0028
8	2.00	0.30	0.06	0.60	7.00	3.29	3.37	0.056	0.579	0.580	0.0007
9	3.50	0.20	0.09	0.76	6.00	5.66	5.52	0.098	0.876	0.872	0.0028
10	5.00	0.30	0.06	0.60	5.00	4.86	4.94	0.056	0.748	0.749	0.0007
11	3.50	0.20	0.09	0.40	6.00	5.88	5.72	0.113	0.890	0.862	0.0198
12	5.00	0.30	0.06	0.20	7.00	4.24	4.32	0.056	0.717	0.718	0.0007
13	5.00	0.10	0.12	0.20	7.00	4.12	4.20	0.056	0.690	0.691	0.0007
14	2.00	0.30	0.12	0.60	5.00	3.24	3.32	0.056	0.570	0.571	0.0007
15	5.00	0.10	0.06	0.60	7.00	4.20	4.28	0.056	0.714	0.715	0.0007
16	0.77	0.20	0.09	0.40	6.00	2.26	2.12	0.098	0.475	0.471	0.0028
17	3.50	0.20	0.09	0.40	6.00	5.40	5.72	0.226	0.870	0.862	0.0056
18	3.50	0.20	0.09	0.04	6.00	5.29	5.15	0.098	0.727	0.723	0.0028
19	3.50	0.20	0.14	0.40	6.00	5.12	4.98	0.098	0.718	0.714	0.0028
20	3.50	0.20	0.09	0.40	4.18	4.08	3.94	0.098	0.660	0.656	0.0028
21	2.00	0.10	0.12	0.60	7.00	3.20	3.28	0.056	0.568	0.569	0.0007
22	3.50	0.20	0.09	0.40	6.00	5.43	5.72	0.205	0.860	0.862	0.0014
23	3.50	0.02	0.09	0.40	6.00	5.25	5.11	0.098	0.722	0.718	0.0028
24	5.00	0.30	0.12	0.20	5.00	4.68	4.76	0.056	0.725	0.726	0.0007
25	3.50	0.38	0.09	0.40	6.00	5.83	5.69	0.098	0.880	0.876	0.0028
26	2.00	0.30	0.12	0.20	7.00	3.18	3.26	0.056	0.546	0.547	0.0007

For identification of A, B, C, D and E refer to Table 1

Table 3 ANOVA, regression analysis and significance of the quadratic model for pullulan production and biomass yield

Source	Pullulan production (%)					Biomass yield ($A_{600}/100$)						
	Coefficient estimate	Sum of squares	F-value	Mean square	df	p-value	Coefficient estimate	Sum of squares	F-value	Mean square	df	p-value
Model/Intercept	5.5800	32.550	28.43	1.6300	20	0.0008	0.8521	0.3727	37.38	0.0186	20	0.0004
A	0.3322	0.7320	12.79	0.7320	1	0.0159	0.0398	0.0105	21.09	0.0105	1	0.0059
B	0.1592	0.1682	2.940	0.1682	1	0.1472	0.0434	0.0125	25.04	0.0125	1	0.0041
C	-0.1620	0.1741	3.040	0.1741	1	0.1417	-0.0448	0.0133	26.65	0.0133	1	0.0036
D	0.1016	0.0684	1.200	0.0684	1	0.3240	0.0409	0.0111	22.27	0.0111	1	0.0052
E	-0.5711	2.1600	37.79	2.1600	1	0.0017	-0.0220	0.0032	6.420	0.0032	1	0.0523
AB	-0.1466	0.0657	1.150	0.0657	1	0.3331	-0.0077	0.0002	0.367	0.0002	1	0.5708
AC	0.1096	0.0367	0.641	0.0367	1	0.4594	0.0711	0.0155	31.03	0.0155	1	0.0026
AD	-0.0889	0.0242	0.422	0.0242	1	0.5445	-0.0028	0.0000	0.047	0.0000	1	0.8367
AE	0.2787	0.2374	4.150	0.2374	1	0.0973	0.0404	0.0050	9.980	0.0050	1	0.0251
BC	-0.3224	0.3176	5.550	0.3176	1	0.0651	-0.0104	0.0003	0.667	0.0003	1	0.4512
BD	-0.5135	0.8057	14.07	0.8057	1	0.0133	-0.0821	0.0206	41.32	0.0206	1	0.0014
BE	-0.0108	0.0004	0.006	0.0004	1	0.9401	-0.0330	0.0033	6.660	0.0033	1	0.0493
CD	-0.2497	0.1906	3.330	0.1906	1	0.1276	0.0008	1.905E-06	0.004	1.905E-06	1	0.9531
CE	0.2579	0.2033	3.550	0.2033	1	0.1182	0.0457	0.0064	12.78	0.0064	1	0.0160
DE	0.0418	0.0054	0.093	0.0054	1	0.7721	-0.0257	0.0020	4.070	0.0020	1	0.0999
A ²	-0.8221	14.010	244.7	14.010	1	<0.0001	-0.0913	0.1728	346.7	0.1728	1	<0.0001
B ²	-0.0156	0.0050	0.088	0.0050	1	0.7785	-0.0149	0.0046	9.210	0.0046	1	0.0289
C ²	-0.0533	0.0589	1.030	0.0589	1	0.3571	-0.0153	0.0049	9.780	0.0049	1	0.0260
D ²	-0.0352	0.0257	0.448	0.0257	1	0.5327	-0.0147	0.0045	9.030	0.0045	1	0.0299
E ²	-0.7694	12.270	214.3	12.270	1	<0.0001	-0.0695	0.1000	200.6	0.1000	1	<0.0001
Residual		0.2862		0.0572	5			0.0025		0.0005	5	
Lack of fit		0.0039	0.055	0.0039	1	0.8254		0.0000	0.077	0.0000	1	0.7946
Pure error		0.2823		0.0706	4			0.0024		0.0006	4	
Cor total		32.840			25			0.3751			25	

For identification of A, B, C, D, E, R1 and R2 refer to Table 1

p-value of the model was significant for R1 and R2

Lack of fit of the model was non-significant for R1 and R2

$$\begin{aligned}
 \text{Biomass yield} = & + 0.8521 + 0.0398A + 0.0434B \\
 & - 0.0448C + 0.0409D + 0.0711AC \\
 & + 0.0404AE - 0.0821BD - 0.0330BE \\
 & + 0.0457CE - 0.0913A^2 - 0.0149B^2 \\
 & - 0.0153C^2 - 0.0147D^2 - 0.0695E^2
 \end{aligned} \quad (3)$$

The lack of fit sum of squares is an important aspect of ANOVA which exhibits the functional relationship between experimental factors and response variables. A lack of fit F -value 0.055 and 0.077 for pullulan production and biomass yield, respectively, depicts the insignificant proportionality of lack of fit to pure error. A non-significant lack of fit F -value is good, which justifies the fitness of present model. The repeated runs in the design with same values for all model terms have a degree of freedom for pure error. Each repeated value contributes one degree of freedom for pure error and the degree of freedom for pure error 3–4 validates the significance of the model. In the present study, degree of freedom for pure error was found to be 4 which validate the authenticity of model. The goodness of fit of model helps in depicting the good correlation between factor's actual and predicted values. A high adequate precision value 17.8115 and 20.2349 for pullulan production and biomass yield, respectively, suggested an adequate signal and confirmed the suitability of present model. The good quality of fitted regression model was demonstrated by value of regression coefficient for pullulan production (0.99) and biomass yield (0.99). A value of R^2 very close to one shows good agreement of predicted and experimental values. Besides, it also demonstrates the reliability of the present model in explaining approximately 100% variability in two responses investigated. The adjusted R^2 value (0.95) for the model was in a good agreement with the predicted R^2 (0.90) and the results are in corroboration with earlier studies (Mehta et al., 2014; Singh et al., 2009b).

Effect of independent factors and their interactions on responses

The effect of independent factors and their interactions on desired responses was investigated using three-dimensional (3D) surface graph plots. The 3D surface plots were obtained by holding three variables at their optimal level, while varying the other two within their experimental range (Fig. 1a, b). The carbon source is an important medium variable and serves as main energy source for proper metabolic activity of *A. pullulans*. It facilitates the cell growth and has a significant role in determining the yield of pullulan. The DRBE sugars were used as sole carbon source during experimental trials. The concentration of

DRBE sugars has shown a significant effect ($p < 0.05$) on pullulan production and biomass yield (Table 3). Maximum pullulan production (5.92%) and biomass yield (0.889 $A_{600}/100$) was obtained, when the production medium was supplemented with 3.5% DRBE sugars. However, further increase or decrease in concentration of DRBE sugars in production medium showed a significant decline in both pullulan production and biomass yield (Fig. 1Aa–d, Ba–d). The main cause of decline in pullulan production at higher or lower sugar concentration may be osmotic effects and lower water activity (Shin et al., 1987). Goksungur et al., (2011) have also reported increase in pullulan production with the increase of initial sugar concentration up to a certain limit only; further increase in sugar concentration significantly lowers the pullulan production. The observations of present investigation are in corroboration with earlier studies on pullulan production by *A. pullulans* (Lazaridou et al., 2002; Singh et al., 2009b). The fermentation medium supplemented with DRBE sugars enhanced the pullulan production by 20% in comparison to a sucrose (pure sugar) based medium reported earlier (Singh et al., 2009b). Moreover, 5.92% (w/v) pullulan production in the present investigation was much higher than the pullulan production 2.4% (w/v) from beet molasses (Lazaridou et al., 2002), 2.51% (w/v) from sugarcane bagasse (Hilares et al., 2019) and 1.87% (w/v) from jackfruit seeds (Sharmila et al., 2013).

The nitrogen supplements facilitate the metabolite production in medium and have strong influence on the polysaccharide production by *A. pullulans*. Yeast extract is composed of peptides, amino acids, vitamins, minerals, etc. and commonly used as a nitrogen source for pullulan production. In present investigation, yeast extract and $(NH_4)_2SO_4$ were used as nitrogen sources in production medium. The linear terms of yeast extract and $(NH_4)_2SO_4$ were observed highly significant ($p < 0.05$) for the biomass yield (Table 3). Maximum pullulan production (5.92%) and biomass yield ($A_{600}/100 = 0.88$) was observed at a minimal amount of 0.20% yeast extract and 0.04% $(NH_4)_2SO_4$ (Fig. 1Aa, b, e, f, Ba, b, e–g). Thus lower amount of ammonium supplements are required for higher yield of pullulan, as high amount of ammonium ions hinder the cell growth of *A. pullulans* and it decreases the exopolysaccharide production (Reed-Hamer and West, 1994). Similar trends have been reported for pullulan production by *A. pullulans* in earlier studies (Lazaridou et al., 2002; Srikanth et al., 2014). In contrary, maximum pullulan production by *A. pullulans* has been reported in a medium supplemented with $(NH_4)_2SO_4$ and NH_4NO_3 as nitrogen source (Thirumavalavan et al., 2009).

Generally, inorganic salts i.e. K_2HPO_4 , KH_2PO_4 , and $(NH_4)_3PO_4$ are supplemented in production medium to fulfill the phosphorus requirement of *A. pullulans* for

Fig. 1 **A** Response surface 3D plots showing the effect of interaction between de-oiled rice bran extract (DRBE) sugars and: **a** yeast extract; **b** ammonium sulphate; **c** dipotassium hydrogen phosphate; **d** pH; effect of interaction between pH and: **e** yeast extract; **f** ammonium sulphate; **g** dipotassium hydrogen phosphate on pullulan production. **B** Response surface 3D plots showing the effect of

interaction between de-oiled rice bran extract (DRBE) sugars and: **a** yeast extract; **b** ammonium sulphate; **c** dipotassium hydrogen phosphate; **d** pH; interaction between yeast extract and: **e** dipotassium hydrogen phosphate; **f** pH; interaction between pH and: **g** ammonium sulphate; **h** dipotassium hydrogen phosphate on biomass yield

Fig. 1 continued

pullulan production. The mineral salts have a significant role in assimilation of carbon and nitrogen sources and are

quite essential for various metabolic activities of a microorganism (Gao et al., 2010). In present investigation,

K_2HPO_4 was used as phosphate supplement. The linear effect of K_2HPO_4 was highly significant ($p < 0.005$) for biomass yield. The combined effect of yeast extract with K_2HPO_4 ($p < 0.05$) was also found to be highly significant for both the responses (Table 3). The biomass yield and pullulan production by *A. pullulans* was increased with increase in concentration of K_2HPO_4 . A concentration of 0.4% K_2HPO_4 was found optimal for maximum pullulan production and biomass yield (Fig. 1Ac, g, Bc, e, h). However, further increase in K_2HPO_4 concentration hinders the cell growth of *A. pullulans*. The results of present study corroborate the data reports in earlier studies (Sharmila et al., 2013; Srikanth et al., 2014).

Mainly pH of the medium is adjusted according to the type of environment required for growth of microorganism, physical parameters of fermentation process and production medium composition. The pH of medium influences the metabolism of *A. pullulans* which further affects pullulan production. It is a crucial factor affecting the morphology of *A. pullulans* (Goksungur et al., 2004). A very low pH (2–4) favors the filamentous morphology, whereas a pH range of 5–6 favors the yeast like morphology (Harvey, 1984). In the present study, the linear and quadratic terms of pH were found significant ($p < 0.05$) for pullulan production and biomass yield. The combined effect of pH with DRBE sugars and $(NH_4)_2SO_4$ also showed high significance ($p < 0.05$) for biomass yield (Table 3). The results established that the mildly acidic pH (5.2) is most favorable for maximum pullulan production and biomass yield (Fig. 1Ad–g, Bd, f–h). Yoon et al., (2012) have also reported the highest exopolysaccharide production by *A. pullulans* between pH 3–5. Our results on mildly acidic pH of the medium as favorable environment for pullulan and biomass production by *A. pullulans* are comparable with earlier reports (Cheng et al., 2009; Li et al., 2009).

Validation of experimental model

The optimal levels of variables i.e. DRBE sugars (3.88%, w/v), yeast extract (0.24%, w/v), $(NH_4)_2SO_4$ (0.06%, w/v), K_2HPO_4 (0.57%, w/v) and pH (5.22) were obtained by experimental trials of generated model. The generated model was validated by performing experiments on shake-flask fermentations using data originated by the designed model. The actual experimental data indicated a pullulan production of 5.48% (w/v) which is in well agreement with predicted value of the model (5.92%, w/v). Similarly, the actual experimental value for biomass yield (0.88) was comparable with the predicted value (0.90). The value of regression coefficient for model was also near to one for both pullulan production and biomass yield. These

observations clearly establish the validity of the generated quadratic model.

Characterization of pullulan

Fourier-transform infrared (FTIR) spectroscopy

FTIR of crude pullulan showed almost similar peaks with pure pullulan (Table 4). The peak at around 850 cm^{-1} indicates the presence of α -configuration in pullulan (Singh and Saini, 2008b). The significant peak at 844.59 cm^{-1} shows the existence α -configuration in crude pullulan structure. The broad peak at around 1019.40 cm^{-1} describes C–O bond vibration of C-4 glucose residue and spectra of crude pullulan showed a comparable peak at 1076.03 cm^{-1} . The band at about 1158.4 cm^{-1} has been assigned to vibrations of C–O–C bond and glycosidic bridge (Kacuráková et al., 2000) and results of crude pullulan have an equivalent peak at 1144.72 cm^{-1} . The structure of crude pullulan was also confirmed with significant peak at 1458.94 cm^{-1} , due to the presence of C–O–H bond. The wave number 3433.36 cm^{-1} signifies the existence of H-bond and similar results (3303.11 cm^{-1}) were observed for crude pullulan. The peak at 2925.38 cm^{-1} represents C–H stretching from pure pullulan is comparable at 2922.78 cm^{-1} peak from crude pullulan. The existence of O–C–O for pure pullulan can be depicted by peaks at 1645.40 cm^{-1} and an analogous peak was observed for crude pullulan at 1646.25 cm^{-1} . The results of present study are in corroboration with earlier findings (Singh and Saini, 2008b; Singh and Kaur, 2018). The slight difference in the structural attributes of crude pullulan in comparison to pure pullulan may be attributed to the presence of impurities in the crude pullulan which might have interfered during the structural analysis.

Table 4 Structural analysis of crude and pure pullulan^a by infrared spectroscopy

Assignment	Wave number (cm^{-1})	
	Crude pullulan	Pure pullulan
H structure	3303.11	3433.36
C–H structure	2922.78	2925.38
O–C–O structure	1646.25	1645.40
C–O–H bend	1458.94	1423.40
C–O–C structure	1144.72	1158.40
C–O structure	1076.03	1019.40
α -configuration	844.59	850.47

^aPure pullulan (Sigma-Aldrich Chemicals Company, USA)

Nuclear magnetic resonance (NMR) spectroscopy

The ^{13}C NMR and ^1H spectra for crude pullulan were almost similar to the pure pullulan. The NMR spectra of crude pullulan confirmed the presence of α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) linkages (ratio 2:1) in its structure (Table 5). The ^{13}C NMR spectra for crude pullulan signified the presence of maltotriose units in a homogenous manner and showed that α -(1 $\rightarrow$ 6) linkage is present between every third glucose ring. The signals at 98.76 ppm for pure pullulan shows the presence of anomeric α -(1 $\rightarrow$ 6) carbon and the nearby signal at 102 ppm was observed for crude pullulan. The shifts for anomeric (1 $\rightarrow$ 4) linked carbon for pure pullulan were at 100.64 ppm or 101.10 ppm and for crude pullulan signals were at 102.72 ppm or 103.01 ppm. The results for structural characteristics of crude pullulan by ^{13}C NMR spectroscopy were in corroboration with earlier study (Singh et al., 2009a). The ^1H NMR spectra gave signals for pure and crude pullulan due to the occurrence of chemical shifts for different linkages in the structure (Table 5). The chemical shift at 5.02 ppm for pure pullulan and 5.25 ppm for crude pullulan signified the presence of anomeric proton at α -(1 $\rightarrow$ 6) linkage. The signals at 5.46 ppm and 5.42 ppm for pure pullulan represented the presence of α -(1 $\rightarrow$ 4) linkage and similar signals were

found for crude pullulan at 5.29 ppm and 5.30 ppm. The ^1H NMR study further confirmed the structure of crude pullulan and the data of present investigation is in corroboration with earlier reports (Singh and Saini, 2008a, b; Singh and Kaur, 2018).

Differential scanning calorimetry (DSC)

The endothermic and exothermic peaks have been observed for crude pullulan. DSC thermogram of pure pullulan showed endothermic transition at 323.51 $^{\circ}\text{C}$ indicating the melting point of pullulan (Fig. 2Ab). DSC thermogram of crude pullulan showed two endothermic transitions between the temperature range 250 $^{\circ}\text{C}$ –280 $^{\circ}\text{C}$ (Fig. 2Aa). The origin of thermal transitions between 250 and 350 $^{\circ}\text{C}$ have appeared due to loss of hydroxyl groups (50–60%) from polymer chain in the form of water molecules (Soppinath and Aminabhavi, 2002). The degradation appeared to be a two-stage process. First endothermic transition showed melting of the pullulan at 221.53 $^{\circ}\text{C}$ with a ΔH of 30.79 J/g and second transition showed at 276.33 $^{\circ}\text{C}$ with ΔH of 49.23 J/g. The first endothermic transition could be due to presence of hydrogen bonding and moisture among O^- moieties and H^+ moieties of galacturonic acid units (Hiorth et al., 2005). The second exothermic transition at 276.33 $^{\circ}\text{C}$ could be due to the degradation of crude pullulan at this temperature. A similar peak has been reported for degradation of pullulan by Einhorn-Stoll and Kunzek (2009).

X-ray power diffraction (XRD)

X-ray diffractograms was conducted to ensure the nature of crude pullulan. The diffractogram with a weak broad peak centered at $2\theta = 19^{\circ}$ indicated that crude pullulan is fully amorphous polymer (Fig. 2Ba). Similar results have been reported for pullulan in earlier findings (Trovatti et al., 2012; Zhang et al., 2013). The XRD of crude pullulan was in agreement with pure pullulan (Fig. 2Bb).

Morphological characteristics

The scanning electron micrographic (SEM) images of crude pullulan at 65X magnification showed discrete particles separated from each other (Fig. 3B). SEM images at higher magnification (800X) exhibited quite smooth surface properties (Fig. 3A). Similar results have been reported earlier (Singh et al., 2018).

In conclusion, the statistical approach was found as an effective methodology for optimization of pullulan production from de-oiled rice bran. Using the optimal media composition, an enhanced pullulan production (5.48%, w/v) and biomass yield (0.88, $A_{600}/100$) were obtained.

Table 5 NMR chemical shifts (in ppm) of carbons and protons in crude and pure pullulan^a at 296 K

Crude pullulan/Pure pullulan ^a			
Carbons	A	B	C
C-1	102.00/98.76	102.72/100.64	103.01/101.10
C-2	73.68/72.48	73.98/72.59	74.00/72.48
C-3	75.55/74.27	76.00/74.18	75.65/73.92
C-4	79.65/78.78	80.01/78.41	79.00/70.38
C-5	72.81/72.38	72.00/72.23	70.99/72.02
C-6	62.50/61.61	61.55/61.32	63.00/67.42
Protons	D	E	F
H-1	5.25/5.02	5.30/5.46	5.29/5.42
H-2	3.69/3.94	3.67/3.72	3.59/3.70
H-3	3.93/4.08	3.89/4.04	3.88/3.79
H-4	3.76/3.76	3.80/3.68	3.70/3.55
H-5	3.72/3.88	3.68/4.01	3.61/4.01
H-6	3.91, 4.69/3.92, 4.01	3.86, 3.82/3.88, 3.96	3.83, 3.57/3.86, 4.00

^aPure pullulan (Sigma-Aldrich Chemicals Company, USA)

A: (1 $\rightarrow$ 4) – (1 $\rightarrow$ 6) – (1 $\rightarrow$ 4)Glc; B: (1 $\rightarrow$ 4) – (1 $\rightarrow$ 4) – (1 $\rightarrow$ 6)Glc

C: (1 $\rightarrow$ 6) – (1 $\rightarrow$ 4) – (1 $\rightarrow$ 4)Glc; D: (1 $\rightarrow$ 4) – (1 $\rightarrow$ 6) – (1 $\rightarrow$ 4)Glc

E: (1 $\rightarrow$ 4) – (1 $\rightarrow$ 4) – (1 $\rightarrow$ 6)Glc; F: (1 $\rightarrow$ 6) – (1 $\rightarrow$ 4) – (1 $\rightarrow$ 4)Glc

Fig. 2 **A** Differential scanning calorimetry of: **a** crude pullulan and **b** pure pullulan. **B** X-ray diffraction patterns of: **a** crude pullulan and **b** pure pullulan

Fig. 2 continued

The use of de-oiled rice bran improved the pullulan production by 20% in comparison to a sucrose based medium (Singh et al., 2009b). This justifies that de-oiled rice bran is

a potential substrate for economical pullulan production. Pullulan was amorphous in nature and its particles have smooth surface.

Fig. 3 Scanning electron micrographic images of crude pullulan at: (A) 65X and (B) 800X

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