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Statistical studies on high molecular weight pullulan production in solid state fermentation using jack fruit seed

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ABSTRACT

The purpose of the work was to optimize the medium variables for maximizing pullulan production using jack fruit seed as a low cost substrate by *Aureobasidium pullulans* in solid state fermentation. Effects of K_2HPO_4, KH_2PO_4, $ZnSO_4{\cdot}5H_2O$, $MgSO_4{\cdot}7H_2O$, NaCl, $(NH_4)_2SO_4{\cdot}5H_2O$, yeast extract, moisture content (%, w/w) in the production medium on pullulan production were studied using Plackett–Burman design. Production of pullulan was significantly affected by the medium variables namely KH_2PO_4, $ZnSO_4{\cdot}5H_2O$, NaCl and moisture content (%, w/w). Then screened variables were optimized by Box Behnken experiment design. The pullulan obtained was characterized and confirmed by FTIR, ^{1}H NMR and ^{13}C NMR. Molecular weight of pullulan was found to be 1.733×10^6 g/mol by gel permeation chromatography (GPC).

1. Introduction

Pullulan is a bio-degradable biopolymer, having potential applications in pharma and food industries (Deshpande, Rale, & Lynch, 1992; Gösungur, Uzunŏgullarí, & Dăgbăglí, 2011). Pullulan, produced by *Auerobasidium pullulans*, consists of uniform repeated units of maltotriose linked by α 1, 4 and α 1, 6 glycosidic bonds (Catley, Ramsay, & Servis, 1986; Sutherland, 1998). Pullulan is colorless, non toxic, odorless, tasteless, non-hygroscopic, water soluble polymer and is insoluble in some organic solvents like ethanol, isopropanol except dimethyl sulfoxide and dimethylformamide (Leathers, 2002; Sugimoto, 1978). It is used as a non calorific food ingredient, food coating in food industry and stabilizing agent, packaging material, coating material, biomaterial in pharmaceutical industry (Alban, Schauerte, & Franz, 2002; Deshpande et al., 1992; Masci, Bontempo, & Crescenzi, 2002; Sivakumar & Rao, 2003).

Agriculture wastes (Israilides, Scanlon, Smith, Harding, & Jumel, 1994; Israilides et al., 1998), jaggery (Vijayendra, Bansal, Prasad, & Nand, 2001), sweet potato (Wu, Jin, Tong, & Chen, 2009), coconut by products (Thirumavalavan, Manikkadan, & Dhanasekar, 2009) jack fruit seed (Sharmila, Muthukumaran, Nayan, & Nidhi, 2013), potato starch waste (Gösungur et al., 2011), cassava starch (Ray & Moorthy, 2007) and palm kernel cake (Sugumaran et al., 2013) have been used as substrate for pullulan production in submerged and/or solid state fermentation.

Pullulan is approximately three times costlier than other exopolysaccharides (Ram, Gaganpreet, & Kennedy, 2008). Solid state fermentation is preferred over submerged fermentation owing to less water consumption, minimum energy requirement and produce high quality product with low cost (Pandey et al., 2000). Media optimization plays a major role in cost of pullulan production. Therefore, medium and process variables are optimized with statistical design of experiments to achieve maximum product yield under low cost (Chakravarti & Sahai, 2003). Initially medium and process variables were screened by fractional factorial design, known as Placket–Burman Design (PBD) (Djekrif-Dakhmouche, Gheribi-Aoulmi, Meraihi, & Bennamoun, 2006; Plackett & Burman, 1946; Yu, Hallet, Sheppard, & Watson, 1997). Response surface methodology (RSM) is an important statistical tool to design the experiments, evaluate interaction between variables and find out optimum conditions for desired response variable (Wang & Lu, 2005). Power of RSM has been successfully exploited in bioprocess applications like metabolites production for optimizing medium and process variables (Gowdhaman, Sugumaran, & Ponnusami, 2012; Latifian, Hamidi-Esfahani, & Barzegar, 2007; Liu, Fang, Lv, Wang, & Chen, 2010). Several researchers had reported pullulan production using statistical design of experiments (Göksungur, Dăgbaăglı, Uçan, & Güvenc, 2005; Jiang, 2010; Singh, Singh, & Saini, 2009). To the best of our knowledge, no article has been reported optimization of pullulan production in solid state fermentation using statistical method.

The objective of the present work was to screen the medium variables, identify the interaction among the variable and to optimize the medium variables for enhancing pullulan production under solid state fermentation using jack fruit seed as a low cost

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solid substrate which contains high carbohydrate and protein content (Bobbio, El-Dash, Bobbio, & Rodrigues, 1978). The pullulan sample was characterized by FTIR, ^{1}H NMR and ^{13}C NMR.

2. Materials and methods

2.1. Microorganism and culture conditions

Yeast like fungal strain, *A. pullulans* NCIM 1049 was obtained from National Chemical Laboratory (NCL), Pune, India. Stock culture was maintained on potato dextrose agar at 4 °C and was sub cultured for every two weeks.

Two loops of *A. pullulans* cells from agar plate were transferred to 250 ml conical flasks containing 100 ml of sterilized seed culture medium containing potato dextrose broth. It was incubated at 30 °C and 200 rpm for 48 h in an orbital shaker. This culture was considered as seed culture and used to inoculate the fermentation media.

2.2. Solid substrate preparation

Jack fruit seed from matured jack fruit (*Artocarpus herterphyllus)* was used as solid substrate for pullulan production in solid state fermentation. Jack fruit seed was collected from local market, Thanjavur, Tamilnadu, India. The collected seed was gently washed with distilled water and dried at room temperature for 3–4 days. Outer layer of the jack fruit seed was removed and seed was cut in to pieces (BSS: +5 and −6).

2.3. Solid state fermentation

Twenty gram of accurately weighed jack fruit seed pieces were taken in 250 ml Erlenmeyer flask which contain known volume of basal medium with the following composition: yeast extract-4.0 (g/l); $(NH_4)_2SO_4$-4.0 (g/l); K_2HPO_4-8.0 (g/l); KH_2PO_4-8.0 (g/l); NaCl-3.0(g/l); $MgSO_4{\cdot}7H_2O$-1.0 (g/l); $ZnSO_4$.$5H_2O$-0.1 (g/l) (Sharmila et al., 2013). The prepared heterogeneous production medium was autoclaved for 15 min at 121 °C. Then the sterilized medium was cooled and then inoculated with 2% (v/v) of 48 h old culture (0.6 OD at 650 nm) of *A. pullulans* grown in seed culture medium.

2.4. Extraction and estimation of pullulan

The pullulan sample from solid state fermentation was added with six volume of ice cool water and kept in an orbital shaker at 200 rpm for 2 h (Ray & Moorthy, 2007). Then sample was then centrifuged for 20 min at 15,000 rpm. In order to ensure efficient separation of particles and cells, centrifugation was carried out twice. Microorganism population in the supernatant was examined by light microscope (Carl Zeiss Inc., Germany). Two volumes of ethanol at 4 °C was added to the supernatant and allowed to precipitate 24 h. The precipitate was then resuspended and centrifuged again at 15,000 rpm for 15 min. Pellet obtained was separated by filtering through pre-weighed Whatman No. 1 filter paper. The filter paper along with the precipitate was dried at 90 °C to constant weight for exo-polysaccharide estimation (Vijayendra et al., 2001). Exopolymer (pullulan) yield was expressed as mg of pullulan per gram of dry substrate (Mitchell, Krieger, & Berovi, 2006).

2.5. Screening of process variables using Plackett–Burman design

A two step experimental design, Plackett–Burman Design (PBD) followed by Box Behnken was carried out to screen and optimize the process and medium variables. PBD was employed to screen statistically significant medium variables and process variables for pullulan production in solid state fermentation. The following eight medium variables namely KH_2PO_4, yeast extract, NaCl, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}5H_2O$ and moisture content were taken in PBD and totally 23 experiments were carried out. Each factor in design was changed at two levels, coded as −1 and +1. This statistical design does not include interaction between the selected process variables and it follows a linear approach for screening of factors. The linear model (Plackett & Burman, 1946) given by Eq. (1) was employed in this design of experiment.

$$Y = \beta_o + \sum \beta_i x_i \tag{1}$$

where Y is the response variable (Pullulan yield in mg/g ds), x_i is the level of independent variable, β_o and β_i are model intercept and linear coefficients, respectively.

In ANOVA analysis, a term is considered to have statistically significant effect on the response variable if its corresponding p-value is less than 0.05. Accordingly terms with p-values less than 0.05 alone were chosen for further optimization studies using response surface methodology (RSM).

2.6. Optimization by response surface methodology (RSM)

Box Behnken design was performed to study the influence of four factors namely, NaCl concentration (g/L), $ZnSO_4{\cdot}5H_2O$ concentration (g/L), K_2HPO_4 concentration (g/L) and moisture content (%, w/w) on response variable, yield of pullulan production in solid state fermentation. Twenty seven experiments including three center points were carried out. For statistical calculations, the medium variable x_i was coded as follows:

$$x_i = \frac{X_i - X_0}{\Delta X} \tag{2}$$

where x_i = coded variable; X_i = process variable; ΔX = Change in uncoded value of a variable. In RSM a quadratic model, as stated in Eq. (3), was adopted to explain the relationship between the response and the independent variables.

$$y = a_o + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1+1}^{n} a_{ij} x_i x_j \tag{3}$$

where y = response variable; a_o, a_{ii}, a_{ij} = regression coefficients.

The adequacy of the regression equation to explain the variation in the response with variations in independent variables was determined by the coefficient of determination R^2. The fitted second order polynomial equation is often represented graphically by contour plot.

2.7. Characterization

The purified sample structure was characterized using FTIR spectroscopy (Perkin-Elmer 1600 spectrophotometer), ^{1}H NMR and ^{13}C NMR spectroscopy (Bruker 300 MHz Instrument, Germany). Sample preparation for FTIR was performed by KBr pellet method (Hui-zhu et al., 2009; Sugumaran et al., 2013; Thirumavalavan et al., 2009). For ^{1}H NMR and ^{13}C NMR, sample preparation was performed by dissolving sufficient amount pullulan obtained from jack fruit seed in DMSO-d6 solvent and TMS was used as an internal standard (Hui-zhu et al., 2009; Lazaridou, Biliaderis, Roukas, & Izydorczyk, 2002; Lazaridou, Roukas, Biliaderis, & Vaikousi, 2002; Sugumaran et al., 2013).

2.8. Molecular weight determination

The average molecular mass of the pullulan sample was estimated using gel permeation chromatography (Agilent 1200 series,

Table 1
Burman factorial design and experimental values of pullulan yield.

Run	KH_2PO_4 (Q, g/L)	Yeast extract (Y, g/L)	$(NH_4)_2SO_4$ (A, g/L)	NaCl (N, g/L)	$MgSO_4{\cdot}7H_2O$ (X, g/L)	$ZnSO_4{\cdot}5H_2O$ (Z, g/L)	K_2HPO_4 (K, g/L)	Moisture (M, %, w/w)	Observed yield (mg/g)	Predicted yield (mg/g)
1	8	0	4	3	0	0	0	30	23.7	27.53
2	8	4	0	3	1	0	0	30	25.21	27.33
3	0	4	4	0	1	0.1	0	30	32.95	30.57
4	0	0	4	3	0	0.1	8	30	19.22	23.02
5	8	0	0	3	1	0	8	70	6.69	7.23
6	8	4	0	0	1	0.1	0	70	17.02	22.72
7	8	4	4	0	0	0.1	8	30	5.07	3.76
8	8	4	4	3	0	0	8	70	2.44	0.94
9	0	4	4	3	1	0	0	70	31.89	27.75
10	8	0	4	3	1	0.1	0	30	35.85	33.77
11	0	4	0	3	1	0.1	8	30	22.59	22.82
12	8	0	4	0	1	0.1	8	70	2.07	2.43
13	0	4	0	3	0	0.1	8	70	15.53	19.96
14	0	0	4	0	1	0	8	70	2.99	3.37
15	0	0	0	3	0	0.1	0	70	50.11	42.83
16	0	0	0	0	1	0	8	30	11.78	10.99
17	8	0	0	0	0	0.1	0	70	28.33	26.81
18	8	4	0	0	0	0	8	30	6.74	0.56
19	0	4	4	0	0	0	0	70	17.63	20.61
20	0	0	0	0	0	0	0	30	28.76	31.47
21	4	2	2	3.5	0.5	0.05	4	50	33.77	32.19
22	4	2	2	3.5	0.5	0.05	4	50	30.37	32.19
23	4	2	2	3.5	0.5	0.05	4	50	32.44	32.19

USA) equipped with a PL gel column of 5 μm pore size (Viscotek, USA) and RI detector. Polystyrene with molecular weights ranging from 200 to 489,300 g/mol was used a standard to construct a calibration curve. DMSO was employed as mobile phase at a flow rate of 0.375 ml/min. One mg/ml of the sample concentration dissolved in eluent was used. The injection volume was 20 μl. All the data processing was carried out by Agilant chem. station software.

3. Results and discussion

3.1. Plackett–Burman design

Pullulan yield (mg/g) under various experimental conditions shown in Table 1 and results were analyzed using Minitab 16. Pareto chart (Fig. 1) illustrates the significance of each factor studied in the investigation. The horizontal bars show the calculated *t*-values, whereas the vertical line represents the table value of 2.19 for 95% level of significance. Students' *t*-values for factors namely yeast extract, $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$ were found to be smaller than the table value indicating that these factors were not statistically significant. The corresponding *p*-value for yeast extract, $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$ were found to be 0.111, 0.061 and 0.662. At 95% confidence level, if the *p*-value of a factor is greater than 0.05 it is considered to be statistically not significant (Göksungur et al., 2005; Gösungur et al., 2011; Hameed, Tan, & Ahmad, 2008; Ponnusami, Krithika, Madhuram, & Srivastava, 2007; Pratibha, Malar, Rajapriya, Balapoornima, & Ponnusami, 2010; Ravikumar, Kim, & Son, 2006).

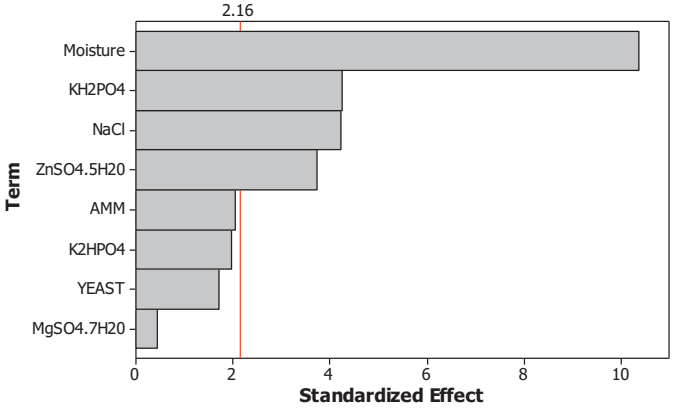

Fig. 1. Pareto chart.

3.2. Response surface methodology

After choosing important medium and process variables influencing pullulan production in solid state fermentation, optimization was done using Box Behnken statistical method. Experimental design employed for pullulan optimization is shown in Table 2. Result obtained from the experiment was fitted to Eq. (3) and regression coefficients along with *p* values are shown in Table 3. Regression coefficients with *p*-values less than 0.05 were taken as a statistically significant at 95% confidence level.

Table 2
Experimental variables at different levels used in RSM and corresponding pullulan yield.

Trial	M	K	Z	N	Observed yield (mg/g)	Predicted yield (mg/g)
1	30	0	0.05	1.5	5.86	5.98
2	70	0	0.05	1.5	2.9	1.75
3	30	8	0.05	1.5	5.06	6.38
4	70	8	0.05	1.5	6.2	6.25
5	50	4	0	0	11.85	11.87
6	50	4	0.1	0	17.78	16.94
7	50	4	0	3	18.43	19.43
8	50	4	0.1	3	18.89	19.04
9	30	4	0.05	0	3.16	3.13
10	70	4	0.05	0	5.92	6.91
11	30	4	0.05	3	15.89	13.92
12	70	4	0.05	3	6.73	5.78
13	50	0	0	1.5	4.71	4.53
14	50	8	0	1.5	21.66	22.08
15	50	0	0.1	1.5	23.37	21.97
16	50	8	0.1	1.5	10.13	9.32
17	30	4	0	1.5	8.89	8.12
18	70	4	0	1.5	5.18	4.66
19	30	4	0.1	1.5	7.88	9.19
20	70	4	0.1	1.5	6.71	8.27
21	50	0	0.05	0	9.93	10.75
22	50	8	0.05	0	14.1	13.12
23	50	0	0.05	3	13.73	15.5
24	50	8	0.05	3	18.06	18.03
25	50	4	0.05	1.5	35.46	37.07
26	50	4	0.05	1.5	36.78	37.07
27	50	4	0.05	1.5	38.98	37.07

Table 3
Regression for full model and reduced model in Box Benhken design.

Term	Full model				Reduced model			
	Coef.	SE Coef.	T	P	Coef.	SE Coef.	T	P
Constant	37.0733	0.9054	40.949	0	37.073	0.9444	39.256	0
M	−1.0917	0.4527	−2.412	0.033	−1.092	0.4722	−2.312	0.034
K	1.2258	0.4527	2.708	0.019	1.226	0.4722	2.596	0.019
Z	1.17	0.4527	2.585	0.024	1.17	0.4722	2.478	0.025
N	2.4158	0.4527	5.337	0	2.416	0.4722	5.116	0
M*M	−19.448	0.679	−28.642	0	−19.448	0.7083	−27.458	0
K*K	−12.532	0.679	−18.456	0	−12.532	0.7083	−17.693	0
Z*Z	−10.060	0.679	−14.817	0	−10.061	0.7083	−14.204	0
N*N	−10.187	0.679	−15.003	0	−10.187	0.7083	−14.383	0
M*K	1.025	0.7841	1.307	0.216				
M*Z	0.635	0.7841	0.81	0.434				
M*N	−2.98	0.7841	−3.801	0.003	−2.98	0.8179	−3.644	0.002
K*Z	−7.5475	0.7841	−9.626	0	−7.548	0.8179	−9.228	0
K*N	0.04	0.7841	0.051	0.96				
Z*N	−1.3675	0.7841	−1.744	0.107				

From the p and t value shown in Table 3, interaction between moisture content and KH_2PO_4, KH_2PO_4 and NaCl, $ZnSO_4{\cdot}5H_2O$ and NaCl, moisture content and $ZnSO_4{\cdot}5H_2O$ were found to be statistically not significant with the p-values greater than 0.05 at 95% confidence level. The p-value of an interaction between moisture content and KH_2PO_4, KH_2PO_4 and NaCl, $ZnSO_4{\cdot}5H_2O$ and NaCl, moisture content and $ZnSO_4{\cdot}5H_2O$ were found to be 0.216, 0.96, 0.107 and 0.434. As a result, these interactions were removed from the regression model. The regression was repeated for the reduced model and results for reduced model are listed in Table 3. It could be seen that all the terms included in this model had statistically significant effect on the response variable pullulan yield which is indicated by the corresponding p-values.

High R^2 value (98.42%) confirmed that the proposed model fitted the experimental data very closely. Further to check the models adequacy, ANOVA analysis was performed and the results are shown in Table 4. The p-values for linear terms, square terms, and interaction terms included in the reduced model were found to be less than 0.05. The p-value for the lack of fit was found to be 0.673 which indicated that lack of fit was not significant and showed goodness of fit. In addition to check the adequacy of the model residual analysis was carried. In the normalized plot (plot not shown here) it was found that the standardized residuals for all the points lie between +2 and −2. Values of regression were substituted in the Eq. (3) (in coded units):

$$\text{Yield } (Y) = 37.073 - 1.092M + 1.226K + 1.17Z + 2.416N - 19.448M^2 - 12.532K^2 - 10.061Z^2 - 10.187N^2 - 2.98MN - 7.548KZ \quad (4)$$

Table 4
ANOVA for pullulan yield in Box Benhken model.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
M	1	14.3	14.3	14.3	5.34	0.034
K	1	18.03	18.03	18.03	6.74	0.019
Z	1	16.43	16.43	16.43	6.14	0.025
N	1	70.04	70.04	70.04	26.18	0
M*M	1	1108.08	2017.27	2017.27	753.94	0
K*K	1	357.14	837.62	837.62	313.05	0
Z*Z	1	266.54	539.84	539.84	201.76	0
N*N	1	553.48	553.48	553.48	206.86	0
M*N	1	35.52	35.52	35.52	13.28	0.002
K*Z	1	227.86	227.86	227.86	85.16	0
Lack of fit	14	36.49	36.49	2.61	0.82	0.673
Pure error	2	6.32	6.32	3.16		
Total	26	2710.22				

The contour plot is shown in Fig. 2. The figure explains the interaction effects of moisture content and $ZnSO_4{\cdot}7H_2O$, KH_2PO_4 and NaCl on pullulan yield. Solving Eq. (4) in an inverse matrix method, optimum values were calculated. The optimum values were: K_2HPO_4-4.16 g/L; moisture content-47.9%; Nacl-1.69 g/L and $ZnSO_4{\cdot}5H_2O$-0.052 g/L. The yield of pullulan was calculated to be 34.22 mg/g under these optimum conditions.

3.3. Characterization of pullulan

3.3.1. FTIR

Fig. 3 shows the FT-IR spectra for pullulan obtained from jack fruit seed. The band appeared at 3388 cm^{-1} and at 2911 cm^{-1} were due to OH stretching and CH stretching, respectively (Sugumaran et al., 2013). Further characteristic signals obtained at 1395, 1177 and 1090 were due to C—OH bending, C—O—C stretching and C—O stretching, respectively. A band appeared at 1639 cm^{-1} is coinciding with that of the previous report for vibrations of the C O C bond and glycosidic linkage (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000; Sugumaran et al., 2013). Other features of the biopolymer were also found from the spectra, O—C—O stretch (1656 cm^{-1}), and C—O—C stretch (1177 cm^{-1}), as reported earlier (Singh & Saini, 2008). A small hump appeared nearly at 848 cm^{-1} was due to α-configuration of α-D-glucopyranose units (Barker, Bourne, Stacey, & Whiffen, 1954). Absorption appeared at 795 cm^{-1} and 968 cm^{-1} were due to α (1, 4) and α (1, 6) linkages between glucose units within pullulan, respectively (Madi, Harvey, Mehlert, & McNeil, 1997).

3.3.2. ^1H NMR

Fig. 4(a) shows the ^1H NMR spectra of pullulan from jack fruit seed. Structural characterization of the purified pullulan from jack fruit seed by *A. pullulans* was carried out by ^1H NMR spectroscopy and it was compared with previous reports. The signals arrived in the downfield region between 4.6 and 5 ppm explained hydroxyl proton (Sugumaran et al., 2013). The signal arrived at 5.003 ppm was due to anomeric proton attached with α (1 → 6) linkage (Ram, Gaganpreet, & Kennedy, 2009). The compound was confirmed as pullulan according to the comparison of the signals obtained with that of commercial pullulan spectra. (Hui-zhu et al., 2009; Ram et al., 2009; Sugumaran et al., 2013).

3.3.3. ^{13}C NMR

Fig. 4(b) shows the ^{13}C NMR spectra of pullulan from jack fruit seed. In ^{13}C spectrum, anomeric α (1 → 6) was indicated by peak resonance at 99.73 ppm (Ram et al., 2009). Splitting of C-4 (76.7and 75.7 ppm) and C-6 (60.4 and 60.3 ppm) resonances of the (1 → 4)

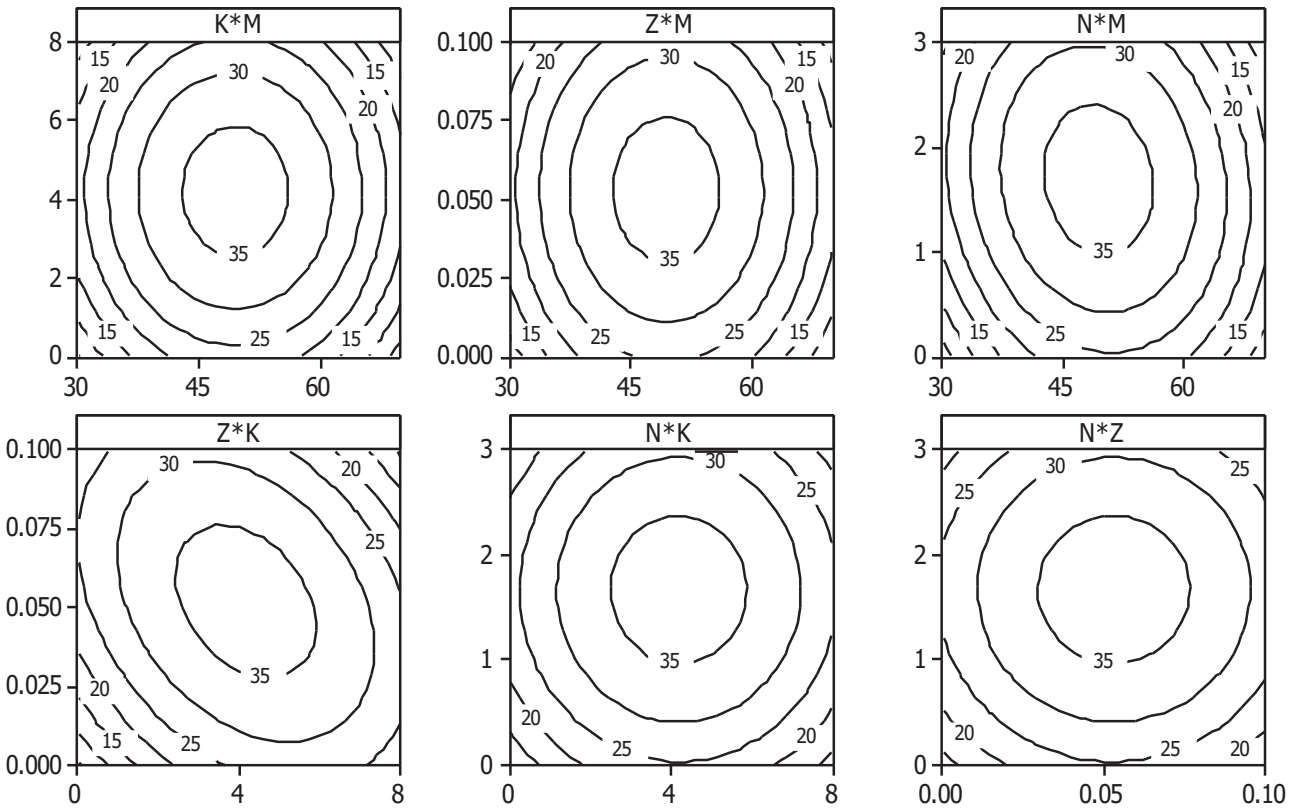

Fig. 2. Contour plots.

linked glucose unit were the result of the sensitivity of carbon positions to the nature of the linkage at C-1 (Lazaridou, Biliaderis, et al., 2002; Lazaridou, Roukas, et al., 2002). C-6 signals arrived at 60.4 and 60.3 ppm are due to two kinds of 1, 4 linked α-D glucose, whereas the signal at 69.3 ppm corresponds to C-6 of the 1, 6 linked α-D glucose (Gorin, 1981; Youssef, Roukas, & Biliaderis, 1999).

3.4. Molecular weight determination

Weight-average (Mw) and number-average (Mn) molecular weights were estimated by gel permeation chromatography. Weight-average (Mw) and number-average (Mn) molecular weights of pullulan from jack fruit seed were found to be 1.1789×10^6 and 3.0475×10^5 g/mol, respectively and polydispersity index was found to be approximately 4. Pullulan molecular weight was mainly affected by factors like medium pH, medium composition, strain *A. Pullulans* and age of inoculumn (Catley, 1979; Israilides et al., 1994; Pollock, Thorne, & Armentrout, 1992; Roukas & Biliaderis, 1995). Average molecular weight (Mw) of commercial pullulan was in the range of 1 to 5.6×10^6 (Lazaridou, Biliaderis, et al., 2002; Lazaridou, Roukas, et al., 2002). Catley (1970) reported that pullulan molecular weight decreased from $3–6 \times 10^6$ to $1–2 \times 10^6$ after 5 days of fermentation (Catley, 1970). Molecular weight of pullulan reduced due to glucoamylase activity in the fermentation medium which catalysis not only starch but also pullulan (West & Strohfus, 1996). Lee and Yoo (1993) reported pullulan molecular weight was dependent on culturing condition and molecular weight of pullulan was found to be in the range from 2

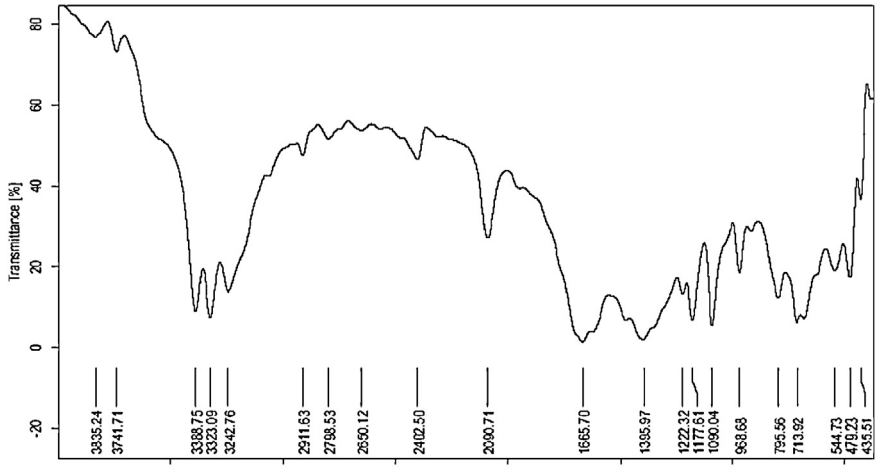

Fig. 3. FTIR for pullulan from jack fruit.

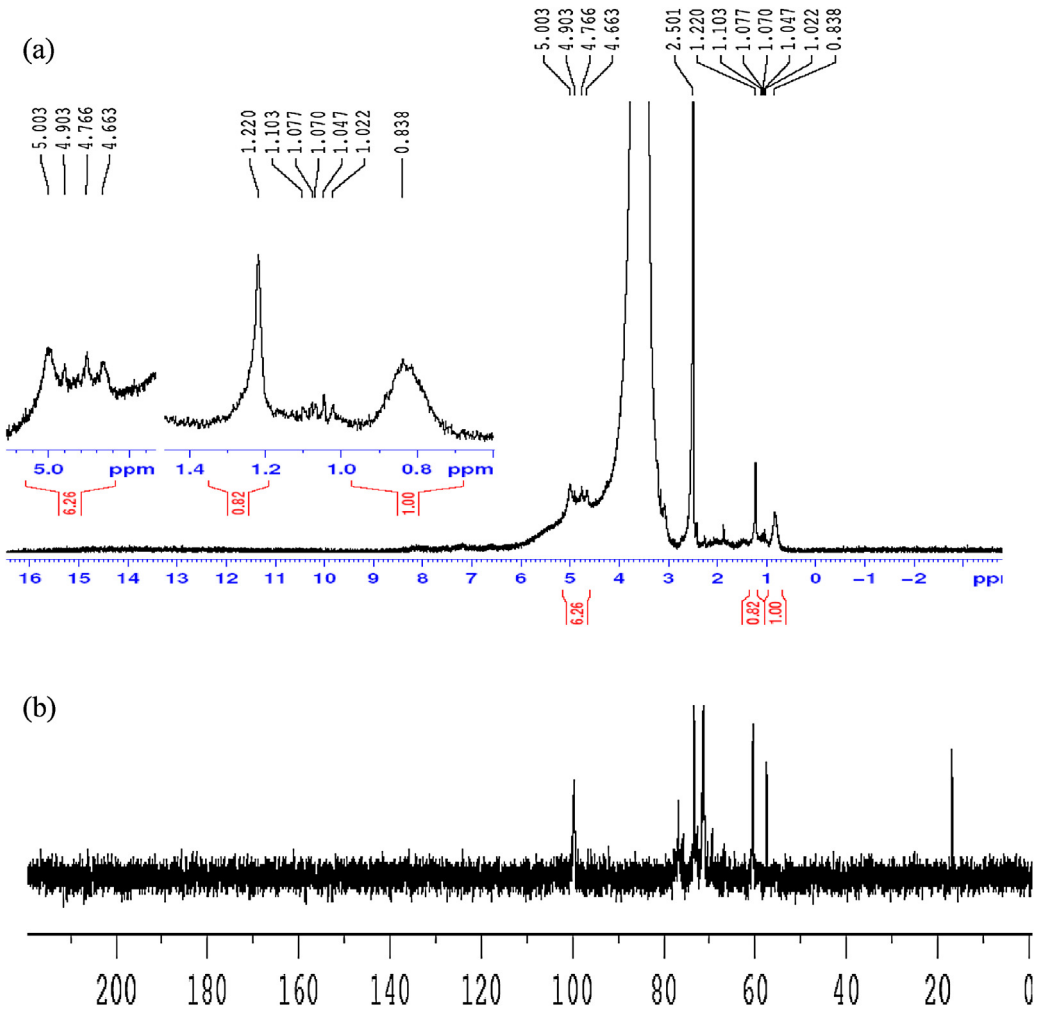

Fig. 4. (a) ^{1}H NMR for pullulan from jack fruit seed and (b) ^{13}C NMR spectra for pullulan from jack fruit seed.

to 6×10^5 (Lee & Yoo, 1993). Lazaridou, Biliaderis, et al. (2002) and Lazaridou, Roukas, et al. (2002) reported high molecular weight of pullulan from beet molasses ranging from 2.07 to 4.06×10^5 (Lazaridou, Biliaderis, et al., 2002; Lazaridou, Roukas, et al., 2002). The molecular weight of pullulan was found to be 2.6×10^5 by *A. pullulans* isolated from sea mud (Wu, Chen, & Pan, 2012).

4. Conclusion

Pullulan production was investigated using jack fruit seed-low cost substrate by *A. pullulans*. Medium variables like KH_2PO_4, $ZnSO_4{\cdot}5H_2O$, NaCl, moisture content (%, w/w) were screened according to Plackett–Burman Design. Then screened variables were optimized by Box Benkhen statistical method. The optimal conditions maximizing the pullulan production (34.22 g/L) were as follows: KH_2PO_4-4.16 g/L, $ZnSO_4{\cdot}5H_2O$-0.052 g/L, NaCl-1.69 g/L, moisture content-47.9%, w/w. Then, purified pullulan was characterized by FTIR, ^{1}H NMR and ^{13}C NMR and compared with previous reports. Molecular weight of pullulan was found to be 1.733×10^6 g/mol by gel permeation chromatography (GPC).

References

Alban, S., Schauerte, A., & Franz, G. (2002). Anticoagulant sulfated polysaccharides: Part I. Synthesis and structure–activity relationships of new pullulan sulfates. *Carbohydrate Polymers*, *47*, 267–276.

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infrared spectra of carbohydrates. Part I. Some derivatives of D-glucopyr-anose. *Journal of the Chemical Society*, *75*, 171–176.

Bobbio, F. O., El-Dash, A. A., Bobbio, P. A., & Rodrigues, L. R. (1978). Isolation and characterization of the physicochemical properties of starch of jackfruit seeds (*Artocarpus heterophyllus*). *Cereal Chemistry*, *55*, 505–511.

Catley, B. J. (1970). Pullulan, a relationship between molecular weight and fine structure. *FEBS Letters*, *10*, 190–193.

Catley, B. J. (1979). Pullulan synthesis by *Aureobasidium pullulans*. In R. C. W. Berkeley, G. W. Gooday, & D. C. Elwood (Eds.), *Microbial polysaccharides and polysaccharases* (pp. 69–80). New York: Academic Press.

Catley, B. J., Ramsay, A., & Servis, C. (1986). Observations on the structure of the fungal extracellular polysaccharide, pullulan. *Carbohydrate Research*, *153*, 79–86.

Chakravarti, R., & Sahai, V. (2003). Optimization of compactin production in chemically defined production medium by *Penicillium citrinum* using statistical methods. *Process Biochemistry*, *38*(4), 481–486.

Deshpande, M. S., Rale, V. B., & Lynch, J. M. (1992). *Aureobasidium pullulans* in applied microbiology: A status report. *Enzyme and Microbial Technology*, *14*, 514–527.

Djekrif-Dakhmouche, S., Gheribi-Aoulmi, Z., Meraihi, Z., & Bennamoun, L. (2006). Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering*, *73*, 190–197.

Göksungur, Y., Dağbaağlı, S., Uçan, A., & Güvenc, U. (2005). Optimization of pullulan production from synthetic medium by *Aureobasidium pullulans* in a stirred tank reactor by response surface methodology. *Journal of Chemical Technology and Biotechnology*, *80*, 819–827.

Gorin, P. A. J. (1981). Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *38*, 13–104.

Gösungur, Y., Uzunõgullarí, P., & Dăgbăglí, S. (2011). Optimization of pullulan production from hydrolysed potato starch waste by response surface methodology. *Carbohydrate Polymers*, *83*, 1330–1337.

Gowdhaman, D., Sugumaran, K. R., & Ponnusami, V. (2012). Optimization of lactic acid production from tea waste by *lactobacillus plantarum* MTCC 6161 in

solid state fermentation by central composite design. *International Journal of ChemTech Research*, *4*(1), 143–148.
Hameed, B. H., Tan, I. A. W., & Ahmad, A. L. (2008). Optimization of basic dye removal by oil palm fibre-based activated carbon using response surface methodology. *Journal of Hazardous Materials*, *158*, 324–332.
Hui-zhu, Z., Fu-ping, G., Ling-rong, L., Xue-min, L., Zhi-min, Z., Xin-du, Y., et al. (2009). Pullulan acetate nanoparticles prepared by solvent diffusion method for epirubicin chemotherapy. *Colloids and Surfaces B: Biointerfaces*, *71*, 19–26.
Israilides, C., Scanlon, B., Smith, A., Harding, S. E., & Jumel, K. (1994). Characterization of pullulans produced from agro-industrial wastes. *Carbohydrate Polymers*, *25*, 203–209.
Israilides, C. J., Smith, A., Harthill, J. E., Barnett, C., Bambalov, G., & Scanlon, B. (1998). Pullulan content of the ethanol precipitate from fermented agro-industrial wastes. *Applied Microbiology and Biotechnology*, *49*, 613–617.
Jiang, L. F. (2010). Optimization of fermentation conditions for pullulan production by *Aureobasidium pullulans* using response surface methodology. *Carbohydrate Polymers*, *79*, 414–417.
Kačuráková, M., Capek, P., Sasinková, V., Wellner, N., & Ebringerová, A. (2000). FT-IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*, *43*, 195–203.
Latifian, M., Hamidi-Esfahani, Z., & Barzegar, M. (2007). Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. *Bioresource Technology*, *98*, 3634L 3637.
Lazaridou, A., Biliaderis, C. G., Roukas, T., & Izydorczyk, M. (2002). Production and characterization of pullulan from beet molasses using a non pigmented strain of *Aureobasidium pullulans* in batch culture. *Applied Biochemistry and Biotechnology*, *97*, 1–22.
Lazaridou, A., Roukas, T., Biliaderis, C. G., & Vaikousi, H. (2002). Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. *Enzyme and Microbial Technology*, *31*, 122–132.
Leathers, T. D. (2002). Pullulan. In E. J. Vandamme, S. De Baets, & A. Steinbuchel (Eds.), *Biopolymers. Polysaccharides II: Polysaccharides from eukaryotes* (pp. 1–35). Weinheim: Wiley-VCH.
Lee, K. Y., & Yoo, Y. J. (1993). Optimization of pH for high molecular weight pullulan. *Biotechnology Letters*, *15*, 1021–1024.
Liu, S., Fang, Y., Lv, M., Wang, S., & Chen, L. (2010). Optimization of the production of organic solvent-stable protease by *Bacillus sphaericus* DS11 with response surface methodology. *Bioresource Technology*, *101*, 7924–7929.
Madi, N. S., Harvey, L. M., Mehlert, A., & McNeil, B. (1997). Synthesis of two distinct exopolysaccharide fractions by cultures of the polymorphic fungus *Aureobasidium pullulans*. *Carbohydrate Polymers*, *32*, 307–314.
Masci, G., Bontempo, D., & Crescenzi, V. (2002). Synthesis and characterization of thermoresponsive N-isopropyl acrylamide/methacrylated pullulan hydrogels. *Polymer*, *43*, 5587L 5593.
Mitchell, D. A., Krieger, N., & Berovi, M. (2006). *Solid-state fermentation bioreactors fundamentals of design and operation* (1st ed.). New York: Springer. Chapter 14.
Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., & Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry*, *31*(2), 135–152.
Plackett, R. L., & Burman, J. P. (1946). The design of optimal multifactorial experiments. *Biometrika*, *33*, 305–325.
Pollock, T. J., Thorne, L., & Armentrout, R. W. (1992). Isolation of new Aureobasidium strains that produce high molecular-weight pullulan with reduced pigmentation. *Applied Environmental Microbiology*, *58*, 877–883.
Ponnusami, V., Krithika, V., Madhuram, R., & Srivastava, S. N. (2007). Biosorption of reactive dye using acid-treated rice husk: Factorial design analysis. *Journal of Hazardous Materials*, *142*, 397–403.
Pratibha, R., Malar, P., Rajapriya, T., Balapoornima, S., & Ponnusami, V. (2010). Statistical and equilibrium studies on enhancing biosorption capacity of *saccharomyces cerevisiae* through acid treatment. *Desalination*, *264*, 102–107.
Ram, S. S., Gaganpreet, K. S., & Kennedy, J. F. (2008). Pullulan: Microbial sources, production and applications. *Carbohydrate Polymers*, *73*(4), 515–531.
Ram, S. S., Gaganpreet, K. S., & Kennedy, J. F. (2009). Downstream processing and characterization of pullulan from a novel colour variant strain of *Aureobasidium pullulans* FB-1. *Carbohydrate Polymers*, *78*(1), 89–94.
Ravikumar, K., Kim, S-H., & Son, Y. A. (2006). Design of experiments for the optimization and statistical analysis of Berberine finishing of polyamide substrates. *Dyes and Pigments*, *75*, 401–407.
Ray, R. C., & Moorthy, S. N. (2007). Exopolysaccharide (pullulan) production from cassava starch residue by *Aureobasidium pullulans strain* MTTC 1991. *Journal of Scientific and Industrial Research*, *66*, 252–255.
Roukas, T., & Biliaderis, C. G. (1995). Evaluation of carob pod as a substrate for pullulan production by *Aureobasidium pullulans*. *Applied Biochemistry and Biotechnology*, *55*, 27–44.
Sharmila, G., Muthukumaran, C., Nayan, G., & Nidhi, B. (2013). Extracellular Biopolymer Production by *Aureobasidium pullulans* MTCC 2195 using Jackfruit Seed Powder. *Journal of Polymers and the Environment*, *21*(2), 487–494.
Singh, R. S., & Saini, G. K. (2008). Pullulan-hyper producing color variant strain of *Aureobasidium pullulans* FB-1 newly isolated from phylloplane of Ficus sp. *Bioresource Technology*, *99*, 3896–3899.
Singh, R. S., Singh, H., & Saini, G. K. (2009). Response surface optimization of the critical medium components for pullulan production by *Aureobasidium pullulans* FB-1. *Applied Biochemistry and Biotechnology*, *152*, 42–53.
Sivakumar, K. A., & Rao, P. (2003). The use of cholesteryl pullulan for the preparation of vincristine pullulan liposomes. *Carbohydrate Polymer*, *51*, 372-322.
Sugimoto, K. (1978). Pullulan production and applications. *Journal of the Fermentation Association, Japan*, *36*, 98–108.
Sugumaran, K. R., Gowthami, E., Swathi, B., Elakkiya, S., Srivastava, S. N., Ravikumar, R., et al. (2013). Production of pullulan by *Aureobasidium pullulans* from Asian palm kernel: A novel substrate. *Carbohydrate Polymers*, *92*(1), 697–703.
Sutherland, L. W. (1998). Novel and established applications of microbial polysaccharides. *Trends in Biotechnology*, *16*, 41–46.
Thirumavalavan, K., Manikkadan, T. R., & Dhanasekar, R. (2009). Pullulan production from coconut by-products by *Aureobasidium pullulans*. *African Journal of Biotechnology*, *8*, 254–258.
Vijayendra, S. V. N., Bansal, D., Prasad, M. S., & Nand, K. (2001). Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77. *Process Biochemistry*, *37*, 359–364.
Wang, Y. X., & Lu, Z. X. (2005). Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by *Boletus* spp. ACCC 50 328. *Process Biochemistry*, *40*, 1043–1051.
West, T. P., & Strohfus, B. (1996). A pullulan-degrading enzyme activity of *Aureobasidium pullulans*. *Journal of Basic Microbiology*, *36*, 377–380.
Wu, S., Chen, J., & Pan, S. (2012). Optimization of fermentation conditions for the production of pullulan by a new strain of Aureobasidium pullulans isolated from sea mud and its characterization. *Carbohydrate Polymers*, *87*, 1696–1700.
Wu, S., Jin, Z., Tong, Q., & Chen, H. (2009). Sweet potato: A novel substrate for pullulan production by *Aureobasidium pullulans*. *Carbohydrate Polymers*, *76*, 645–649.
Youssef, F., Roukas, T., & Biliaderis, C. G. (1999). Pullulan production by a nonpigmented strain of *Aureobasidium pullulans* using batch and fed-batch culture. *Process Biochemistry*, *34*, 355–366.
Yu, X., Hallet, S. G., Sheppard, J., & Watson, A. K. (1997). Application of the Plackett–Burman experimental design to evaluate nutritional requirements for the production of Colletotrichum coccodes spores. *Applied Microbiology and Biotechnology*, *47*, 301–305.