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Strain Improvement and Statistical Analysis of Pullulan Producing Strain of *Aspergillus japonicus-VIT-SB1* for Maximum Yield

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In this study, we have screened a mutant strain of *Aspergillus japonicus-VIT-SB1* by UV rays and EMS mutagenesis. The resulted mutant strain showed an increased molecular weight, less pigmentation with hyper production of pullulan as compared to parent strain. The various physical parameters were optimized and it was found that incubation temperature 35°C, rpm 200, incubation time 7 days, initial pH 6 and the inoculums size of 10×10^6 were the optimum condition for the maximum production of pullulan. Considering these optimized parameter, a response surface methodology was employed to understand the interactions among the components of the complex media with second order quadratic model. Results indicated that the concentration of peptone has significant influence in pullulan production as compared with yeast extract. Understanding the effect of complex nitrogen sources in the media using statistical methods has resulted in 73.63 g/L pullulan production which is higher as compared to earlier reports.

Key words: *Aspergillus japonicus*; Central Composite Design; High Molecular weight; Optimal condition; Pullulan.

Fermentative production of pullulan has been widely studied in recent years. The polymers synthesized by microorganisms have received increasing interest, mainly because of their useful physiochemical nature and uncomplicated biodegradability properties within the natural conditions. Because of its distinctive physical and chemical properties, pullulan contains a wide range of commercial and industrial applications in several fields together with the food and cosmetic industries, environmental treatment, pharmacy and healthcare, and even lithography[1,2]. Throughout batch or fed-batch fermentation process, the yield of pullulan with respect to substrate is a key indicator of pullulan production in a cost effective way. In fact very little studies have targeted on these aspects so far. In addition production of melanin pigments along with pullulan create a major problem with regard to its purification process[3] . Hence it is very necessary to find a microbial strain which is deficient in pigment production for maximum yield.

In the recent review on the pullulan, it is reported that the cost of pullulan is about three times higher than that of other polysaccharides. So many approaches were taken together in order to reduce the cost of pullulan production, which includes, potential strains selection[4] , selection of effective carbon and nitrogen sources[5] and engineering innovations for the development and designing of fermenter. The media components which are generally used for the production of pullulan accounts 30% of total production cost[6] . Potato starch waste[7] , deproteinized whey[8] ,

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coconut by-products[9] , Agro-industrial wastes[10,11] , jaggery[12] , sweet potato[13] , brewery wastes[14] , corn steep liquor[15] , beet molasses[16] and hydrolyzed potato starch waste[17] were the various alternate carbon sources reported in pullulan literature.

In the present study, we began by screening a pigment-free and high molecular weight pullulan producing strain by both physical (UV ray) and chemical (EMS) mutagenesis. Secondly, detailed studies on the optimization of different physical parameters for maximum yield were carried out using single point optimization study. Further, by using second order quadratic model and central composite design a three dimensional response surface analysis was done in order to find the effect of different media components for the enhancement of pullulan production. This study is the first detailed work on optimization of different physical parameters and media components for maximum yield of pullulan from the mutant strain of *Aspergillus japonicus-VIT-SB1*.

MATERIALS AND METHODS

Complex Mutagenesis for the Strain Improvement

Aspergillus japonicus-VIT-SB2, a strain deficient in pigment production, is a mutant of the melanin pigment producing strain, *Aspergillus japonicus-VIT-SB1* (GenBank Accession No: KC128815) which had been isolated from from the Amirthi forest (12.9165167 N, 79.13249859 E, Located in the Javadi Hills of Tellai across Amirthi River, 25 km from the Vellore city) Tamil Nadu, India.

For the analysis of survival rates by UV mutagenesis, cells grown for 24 h at 28°C on a YPD (1% yeast extract, 2% peptone, 2% glucose and, if necessary, 2% agar) medium plate were collected and suspended in sterile water. After the cell concentration was determined by counting, cells were spread on YPD medium plates. The plates were placed under a UV lamp (Toshiba GL15) at a distance of 30 cm in aseptic condition and were irradiated for various periods of time. Following irradiation, the plates were incubated at 28°C, and the numbers of colonies were counted to determine survival rates[18]. For the analysis of survival rates by EMS (Ethyl Methane Sulfonate) mutagenesis, cells were inoculated into liquid YPD medium and grown for 24 h at 28°C. The cells were washed once with sterile water, diluted, and suspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0). To the suspension, 30µl of EMS was added. After various incubation times, the cells were washed once with 1 ml of 5% sodium thiosulfate, suspended in water, and spread on YPD medium plates to determine survival rates[19] . The cells with a survival rate of about 25% were plated on a commonly used medium[20] and incubated at 30°C for 2 days. Colonies without pigment were isolated and from these isolated colonies the strain with the highest pullulan production was screened .The mutant strain *Aspergillus japonicus-VIT-SB2* was used throughout this study for the production of pullulan.

Improvement of Cultural Conditions with Optimal Physical parameters

Various optimal physical parameters required for maximal Pullulan production by *Aspergillus japonicus-VIT-SB2* in a shake flask system were studied. These included different ranges of temperature (25°C, 30°C, 35°C, 40°C, 45°C, and 50 °C), agitation speed (0, 50, 100, 150, 200, and 250 rpm), incubation time (1st day, 2nd day, 3rd day, 4th day, 5th day, 6th day, 7th day, 8th day and 9th day), initial pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0) and inoculums sizes (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8). Unless stated otherwise, the initial fermentation medium consisted of 50 g/L sucrose, 2.5 g/L yeast extract, 5.0 g/L K_2HPO_4, 0.6 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4\cdot7H_2O$, and 1.0 g/L NaCl . All experiments were carried out in triplicate and the values were reported as standard deviations. Seed cultures were prepared by inoculating cells in a 500 ml Erlenmeyer flask containing 50 ml YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and incubated at 30°C for 24 h on a rotary shaker at 200 rpm. The seeds were then transferred to 500 ml flasks containing 50 ml of fermentation media. Flask fermentation was carried out initially with pH 5.0, temperature 30°C, rpm 200 and inoculums size of 5×10^6. After optimization of the above physical parameters, the batch fermentation for pullulan production was conducted in a 5 litre stirred fermentor (Lark Innovative Fermentor) containing 3 litre of fermentation media.

Analytical methods

Fermentation broth (25 ml) was heated at 80°C for 15 min in a water bath, and then centrifuged at 8000 rpm for 20 min to remove cells after being

cooled to room temperature. The biomass was determined by drying the cells at 70°C to a constant weight. Supernatant polysaccharides were precipitated with 2 volumes of ethanol at 4°C for 12 h. The precipitate was centrifuged at 8000 × g and 4°C for 20 min followed by drying at 80°C overnight and then weighed[21,22] and the potentiality of this strain to produce pullulan was checked by FT-IR, and enzymatic (Pullulanase Hydrolysis) assay as given by Singh et al. 2010 [28]. Residual sugar concentrations in the supernatant were determined using the dinitro salicylic acid (DNS) method[23].

The limiting viscosity of pullulan aqueous solutions with deionized water was calculated by using an Ubbelohde capillary viscometer. The intrinsic viscosity [η] was determined from the Huggins equation i.e $[\eta] = (0.000258) \times Mw^{0.646}$ and the average molecular weight (Mw) of pullulan was calculated from this equation[24] .

The melanin pigment estimation of the parent strain and mutatnt strain were estimated by the standard method[25,26] .

Central Composite Design (CCD) with RSM model for optimization

Response surface methodology (RSM) was used to study the interaction among complex media components and their contribution towards pullulan production. The Central Composite Design (CCD) with 3 factors and 5 levels including 3 replicates were made for the establishment of second order 3-dimensional response surface with the help of Design-Expert 7.0. The CCD was developed as an embedded factorial matrix with center points and star points pointed (replicate of axial point) around the center point which allows to analyse the curvature. The star points represent the extreme values (both low and high) for each factor in this design and hence in case of full factorial design α is equal to $(2K)^{1/4}$. In our case K is equal to 3 i.e. sucrose, yeast extract and peptone, hence value of α will be1.68179. Each variable was studied into five different levels and these are listed in Table 2. According to this, a set of 20 experiments which include 6 center points, 6 axial points with α value 1.68179 were carried out. A multiple regression analysis of the data was carried out and the second-order polynomial equation that defines predicted response (Y) in terms of independent variables was obtained:

$$Y = X_o + X_1A + X_2B + X_3C + X_{11}A_2 + X_{22}B_2 + X_{33}C_2 + X_{12}AB + X_{13}AC + X_{23}BC$$

where Y represents response of variables, X_o is intercept coefficient, X_1, X_2, X_3 are linear coefficients, X_{11}, X_{22}, X_{33} are squared coefficients and X_{12}, X_{13}, X_{23} are the interaction coefficients. Combination of factors (such as AB, AC and BC) represent an interaction between the individual factors in that term. Statistical analysis of the model was performed to evaluate the analysis of variants (ANOVA) after obtaining values of response by carrying out experiments as suggested by the model. Statistical significance of the model equation was determined using Fisher's test value (F-value) and proportion of variance explained by the model which was indicated by R^2 value. For each variable the quadratic model was represented by contour plot and the 3-dimensional response surface plot were generated to understand the effect of variables individually and in combination. These plots were also used in determining the optimum media composition for obtaining higher production of pullulan. All the above statistical analysis was performed with the help of Design-Expert 7.0 .

To validate the predicted optimum media composition shake flask fermentation was carried out using a media containing sucrose, yeast extract and peptone in triplicates. Concentration of media components was varied according to the experimental design. In all cases a 250 ml conical flask containing 50 ml of media was inoculated with 5% (v/v) inoculums and incubated at 28 °C in a rotary shaker with 250 rpm for 96h.

RESULTS AND DISCUSSION

Complex Mutagenesis for the Strain Improvement

Melanin pigment is an obstacle to pullulan industrial production because it increases pullulan recovery and purification costs[27]. Hence, a strain deficient in pigment formation for efficient pullulan production is necessary. Complex mutagenesis of *A. japonicus-VIT-SB1* by UV ray and EMS treatment were conducted and the results are listed in Table 1. After the treatment, a non-pigmented strain was screened which was designated as *A. japonicus-VIT-SB2* .

The *Aspergillus japonicus-VIT-SB2* strain could able to produce the high molecular

weight ($2.1\pm0.08\times10^6$) and pigment free pullulan with higher yield (1.43 times more) with respect to the parent strain.

Analytical methods

The structural characterization of the Pullulan obtained from *Aspergillus japonicus-VIT-SB2* by IR spectroscopy yielded spectra similar to that of the Pullulan (Sigma, USA) which is given in Table 2.The HPLC chromatogram has shown that the sample peak was matched with a standard pullulan obtained from Sigma. The percentage of hydrolysis with production of reducing sugar at 150 rpm in specific time intervals shows that maximum 93.66 % of pullulan hydrolysis was achieved after 280 min yielding 4.78 mg/ml of reducing sugars (Fig. 1). These results depict that the EPS used as a substrate for the Pullulanase enzyme was Pullulan

Optimization of Physical parameters to improve the culture condition

Effect of Temperature on Pullulan production

A study was conducted to investigate the effect of temperature varying from 25°C to 50°C. Maximum pullulan production was achieved at a temperature of 35°C as shown in Fig. 2. In contrast, other reports have described optimal conditions for pullulan production as temperature 24 °C in the fermentation with *Aureobasidium pullulans* [28,29]. The different optimal temperature conditions reported in the literature may be due to the differences in the types of strain, composition of fermentation medium and culture conditions

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

Strains	Pigments	Pullulan (g/l)	Melanin Pigment Concentration (g/l)	Mw ($\times10^6$)	Colony Morphology
Aspergillus japonicus-VIT-SB1(Parent Strain)	Dark Pigmentation	39±0.02	12±0.02	1.9±0.11	
Aspergillus japonicus-VIT-SB2(Mutated Strain)	Light Pigmentation	56±0.02	2.2±0.02	2.1±0.08	

Table 2. Comparative infra-red spectroscopy data of Pullulan produced from *Aspergillus japonicus-VIT-SB2* with standard pullulan (Sigma, USA)

Assignment	Pullulan from Sigma (Wave Number in cm^{-1})	Pullulan obtained from *Aspergillus japonicus-VIT-SB2* (Wave Number in cm^{-1})
O-H str.	3432.4	3452.58
C-H str.	2927.3	2924.09
O-C-O str.	1639.7	1641.42
C-O-H bend	1366.4	1384.89
C-O-C str.	1154.9	1157.23
C-O str.	1021.4	1087.85

Table 3. Experimental range of the variables studied using CCD in terms of coded and actual factors

Variable	Symbol	Coded Level				
		-1.682	Low(-1)	Mid(0)	High(+)	+1.682
Sucrose	A	11.29	13	15.5	18	19.70
Peptone	B	0.32	1.00	2.00	3.00	3.68
Yeast extract	C	0.49	1.00	1.75	2.5	3.01

used[30]. However, cell growth of *Aspergillus japonicus-VIT-SB2* increased with temperature in the range of 25-30°C. The maximum level of biomass production was found to be at 30°C. Therefore, the optimal temperature of cell growth is also not in accordance with that of pullulan production.

Effect of RPM on Pullulan production

In the submerged cultures, agitation speed was found to be a critical factor influencing both the fungal mycelial biomass and pullulan

Table 4. Experimental designs used in RSM studies to understand interaction among media components for pullulan production

Std Order	Run	Sucrose (gm/L)	Peptone (gm/L)	Yeast Extract (gm/L)	Mean Observed Response of Pullulan (gm/L)	Predicted Response of Pullulan(gm/L)
1	2	18	1	1	21.81	21.62
2	16	15.5	2	1.75	58.2	56.79
3	15	15.5	2	1.75	56.49	56.79
4	7	13	3	2.5	58.5	60.61
5	11	15.5	0.318207	1.75	14.72	15.35
6	12	15.5	3.681793	1.75	73.63	70.27
7	19	15.5	2	1.75	59.27	56.79
8	17	15.5	2	1.75	56.61	56.79
9	13	15.5	2	0.488655377	48.66	45.79
10	5	13	1	2.5	43.74	43.07
11	1	13	1	1	19.24	20.94
12	8	18	3	2.5	66.61	66.83
13	18	15.5	2	1.75	54.12	56.79
14	9	11.29551792	2	1.75	49.34	47.05
15	14	15.5	2	3.011344623	62.1	62.25
16	3	13	3	1	60	62.27
17	20	15.5	2	1.75	55.58	56.79
18	6	18	1	2.5	43.2	42.85
19	10	19.70448208	2	1.75	53.28	52.85
20	4	18	3	1	66.78	69.38

Table 5. Analysis of variance (ANOVA) for the all terms of model

Source Model	Sum of Squares	Degree of freedom (DOF)	Mean Square	F Value	p-value Prob > F	
	4719.383519	9	524.3759465	84.2745171	< 0.0001	Significant
A-Sucrose	40.59694323	1	40.59694323	6.52449413	0.0287	Significant
B-Peptone	3640.486244	1	3640.486244	585.076837	< 0.0001	Significant
C-Yeast Extract	326.9678916	1	326.9678916	52.5482935	< 0.0001	Significant
AB	20.67245	1	20.67245	3.3223506	0.0983	
AC	0.39605	1	0.39605	0.06365075	0.8059	
BC	282.7442	1	282.7442	45.4409304	< 0.0001	Significant
A^2	84.33724945	1	84.33724945	13.5541705	0.0042	Significant
B^2	351.9334916	1	351.9334916	56.5606131	< 0.0001	Significant
C^2	13.84519464	1	13.84519464	2.22511559	0.1666	
Residual	62.22236149	10	6.222236149			
Lack of Fit	45.40527815	5	9.08105563	2.69994964	0.1498	Not significant
Pure Error	16.81708333	5	3.363416667			
Cor Total	4781.60588	19				

production. Fig. 3 shows that pullulan production was increased up to the 200 rpm, where it was achieved maximum (38.05 g/L) and after that the rate of production get decreased. Additionally, changes in the morphology of *Aspergillus japonicus-VIT-SB2* caused by different agitation speeds may influence growth of the microorganism.

Time course of Fermentation

Kinetics studies on the production of pullulan by *Aspergillus japonicus-VIT-SB2* were made for a period of 9 days. There was a slight increase in the production of pullulan with biomass over each days. Maximum pullulan yield was observed after 7 days. However, the biomass increased during the whole experimental period (Fig. 4) and it decreased after 7 days of the fermentation process. So it is evident from the results that the production of pullulan by *Aspergillus japonicus-VIT-SB2* is a growth associated process. But in some of the earlier report, it was found that pullulan production is a non-growth associated process[29] . The increase in pH may be due to switch of culture's metabolism from sugar utilization to protein decomposition.

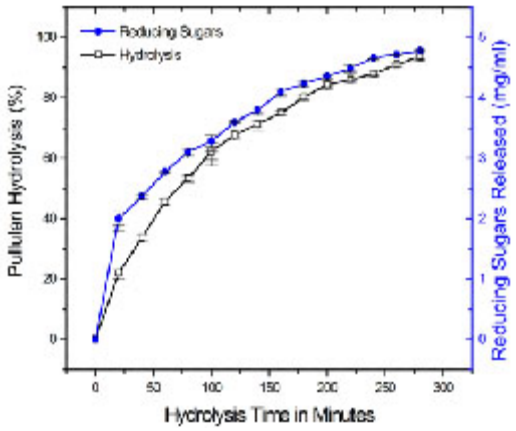

Fig. 1. Hydrolysis of EPS obtained from *Aspergillus japonicus-VIT-SB2* strain by Pullulanase (Source: *Bacillus acidopullulyticus*, Sigma, USA) enzyme with simultaneous release of reducing sugars (Values were representative of three separated experiments)

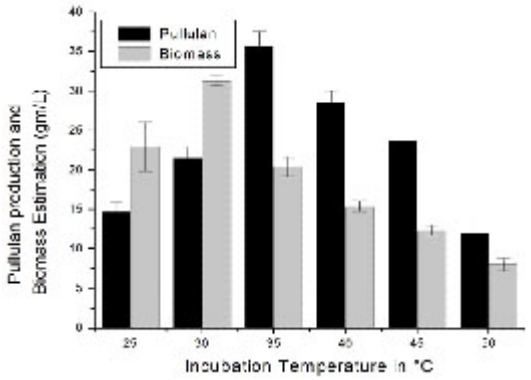

Fig. 2. Effect of Temperature on pullulan production and cell growth. Data are shown as Mean ± SD (n = 3)

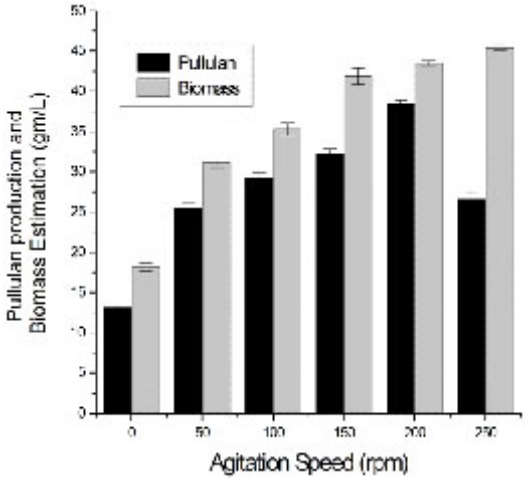

Fig. 3. Effect of Agitation Speed (rpm) on pullulan production and cell growth. Data are shown as Mean ± SD (n = 3)

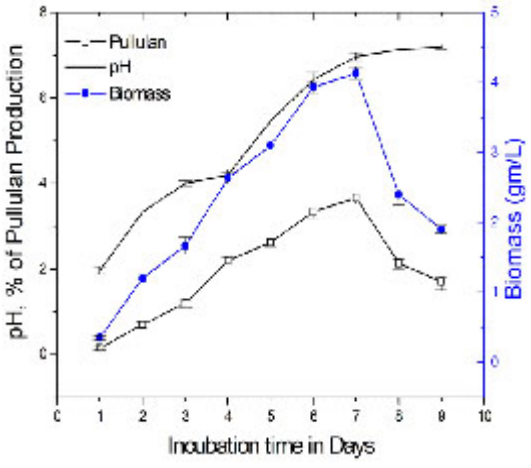

Fig. 4. Effect of incubation time on pullulan production and cell growth. Data are shown as Mean ± SD (n = 3)

First, culture grows quickly on sugar as it doesn't need any 'splitting'. Then, when sugar source is exhausted the culture switches to protein source.

Effect of initial pH on Pullulan production

Effect of initial pH values ranging from 3 to 9 in the media on pullulan production by the microorganism was investigated and the results were shown in Fig. 5. Yeast-like cell was observed at all the initial levels of pH, and relatively low pH is suitable for biomass growth . Maximum pullulan production (37.33 g/L) in the medium broth was observed at an initial pH of 6 (Fig. 5) . In contrast to our results, several previous reports indicated that the optimal pH values for pullulan production were obtained at initial pH of 5.0 , 6.5 and 7.5 [12,24,31]. The different optimum initial pH conditions reported may be related to function of the different types of species, medium composition, and fermentation conditions used.

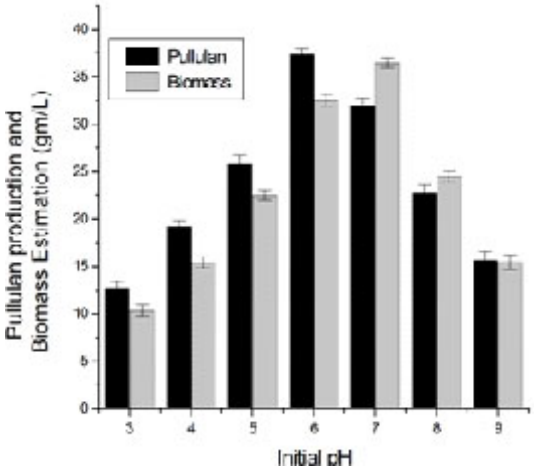

Fig. 5. Effect of Initial pH on pullulan production and cell growth. Data are shown as Mean ± SD (n =3)

Effect of inoculums size on Pullulan production

Inoculum size is another important parameter for pullulan production and the size of inoculum has been reported to play a significant role in the production of metabolites. *Aspergillus japonicus-VIT-SB2* pellets were observed at all the different level of spore inoculum studied, however, studies on the effect of inoculum's size on pullulan production showed enhanced production with increasing inoculum's sizes up to spore concentrations in the range of 1×10^5 to 1×10^6 spore/mL (Fig. 6A). In order to determine the actual inoculums size that gave maximal pullulan production the spore concentration was narrow down to 2×10^6 to 12×10^6 spores/mL (Fig. 6B). The results revealed from Fig. 6A and Fig. 6B showed that the maximal pullulan production of (36.2 gm/mL) was obtained at the spore concentrations of about 10×10^6 spores/mL . However, there was not much different in pullulan production at the inoculums size between 6×10^6 to 10×10^6 spores per mL. The results obtained suggested that lower concentration of inoculums size may have not been

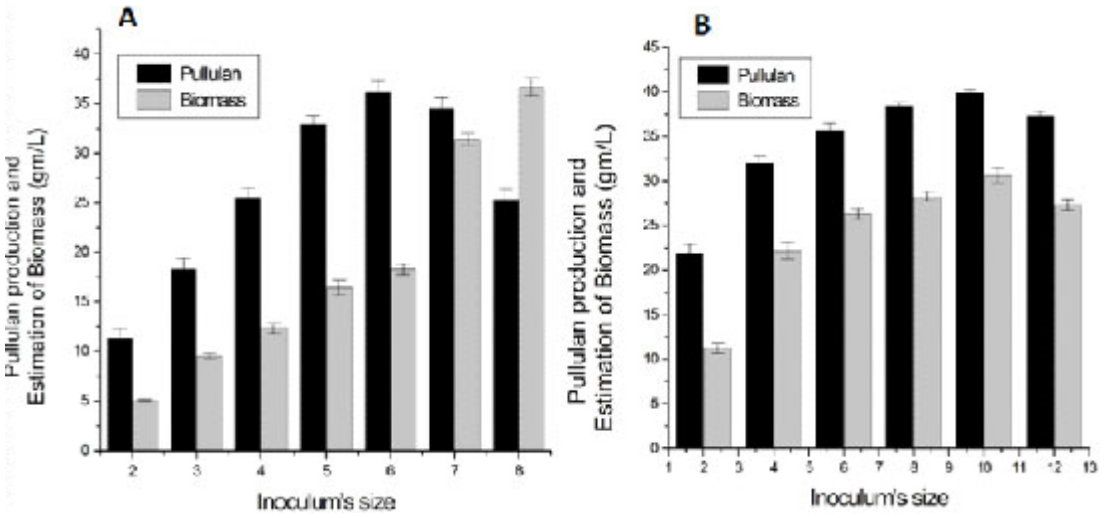

Fig. 6. Effect of inoculum's size on biomass and Pullulan production by cells of *Aspergillus japonicus-VIT-SB2* in a shake-flask system. (A) Wide range (1×10^X Spores/mL), (B) Narrow range ($\times10^6$ Spores/mL) . Data are shown as Mean ± SD (n = 3)

sufficient for initiating growth and pullulan synthesis and therefore, an increase in the number of spores ensured a rapid production of biomass and biosynthesis of pullulan. However, the decreased in pullulan production after an inoculum's size of about 10×10^6 spores per mL was presumably because of the depletion of nutrients due to enhanced biomass production.

Experimental design and optimization with laboratory scale fermenter

The Central Composite Design was employed with the help of Design-Expert 7.0. software which is a very useful tool to determine the optimal level of media composition and their interaction. Different levels at which media components (Sucrose, Peptone and Yeast extract) were varied for pullulan production is shown in Table 3.

The results of CCD experiments with different media composition are presented in Table 4 along with observed and predicted response. The initial evaluation of the design shows a quadratic model will be suitable for the purpose. This model has 9 degrees of freedom with 5 degrees of freedom for lack of fit and 5 degrees of freedom for pure error. This data ensures the valid lack of fit test. Statistical testing of the model was done by the Fisher's statistical test ('F' Test) for analysis of variance (ANOVA) and the result is tabulated in Table 5. The data was fitted with second-order polynomial function (Eq. 1).

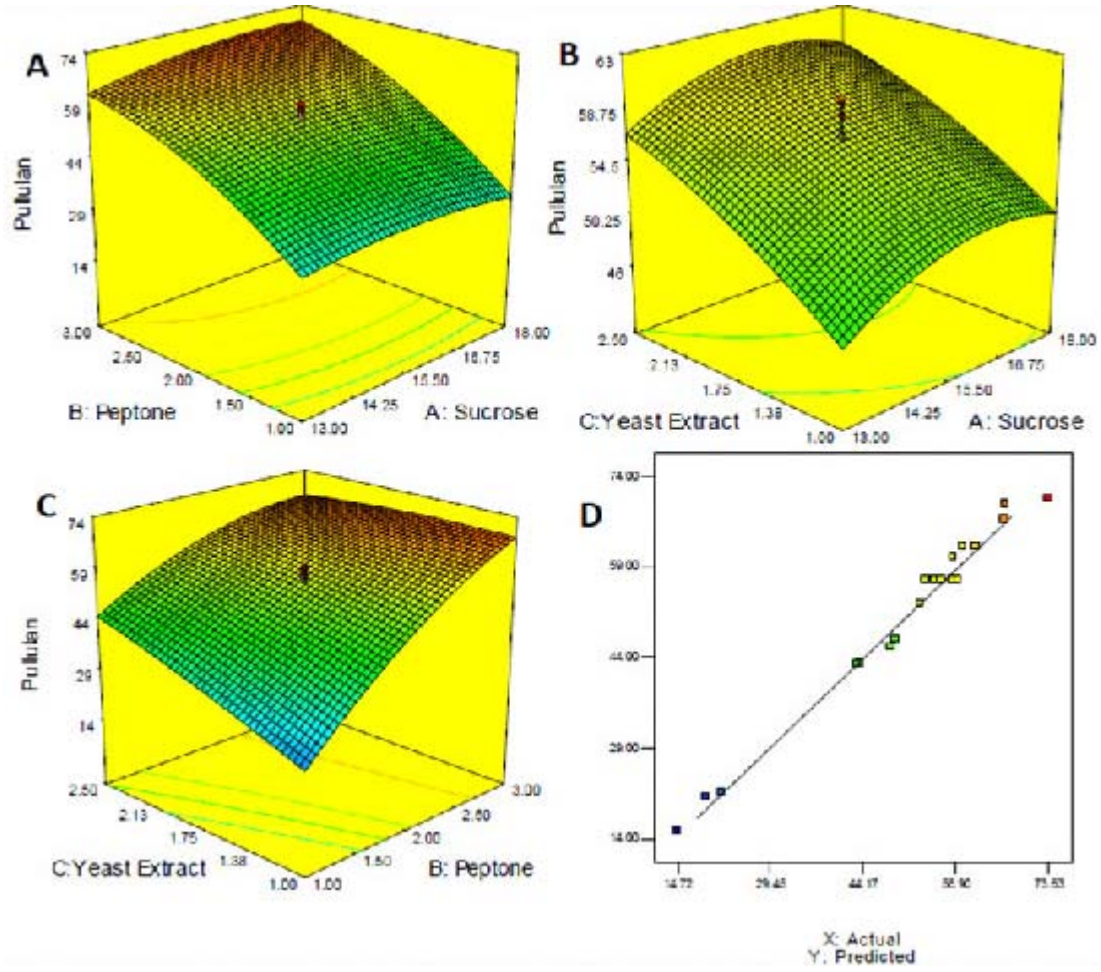

Fig. 7. Three dimensional response surface plot to find the interaction between peptone and sucrose (A), yeast extract and sucrose (B) yeast extract and peptone (C) for maximum production of pullulan and the Parity plot showing the relation between actual and predicted values for pullulan elaboration (D)

The analysis of variance (Table 5) for the model F-value of 84.27 implies that the model is significant.There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant.In this case A, B, C, BC, A^2, B^2 are more significant model terms.Values greater than 0.1000 indicate the model terms are not significant.If there are many insignificant model terms (not counting those required to support hierarchy), model reduction can able to improve the model. The "Lack of Fit F-value" of 2.70 implies the Lack of Fit is not significant relative to the pure error (Non-significant lack of fit is good). There is a 14.98% chance that a "Lack of Fit F-value" may be large which occures due to noise in the model.

It is known that R^2 value greater than 0.75 indicates the aptness of model. In our case, the regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.9870 which shows that this model can explain 98.70% variation in the response. The "Predicted R^2 Value" of 0.9222 is in reasonable agreement with the "Adjusted R^2 Value" of 0.9753. Therefore, the model is expected to predict the response more correctly in the present case. The "Adeq Precision" analysis in the model measures the signal to noise ratio. A ratio greater than 4 is desirable.Here in this model the signal to noise ratio is 31.135, which indicates an adequate signal.The coefficient of variation (CV) indicates the degree of precision with which experiments were compared. High CV value indicates lower reliability of experiments, in our case lower value of CV i.e 4.88 indicated the greater reliability of the performed experiments.

With this above informations, this model can be used to navigate the design space.

$$\text{Pullulan} = 56.79 + 1.72A + 16.33B + 4.89C + 1.61AB - 0.22AC - 5.94BC - 2.42A^2 4.94B^2 - 0.98C^2 \quad \text{(Eq. 1)}$$

Whereas A, B and C are Sucrose, peptone and yeast extract respectively.

Optimization of media component with Response Surface Methodology

The response surface and contour plots are graphical representations of the regression equation developed. These three dimensional plots were used to understand the interaction between different media components and also to determine the optimum level of each component required for significant production of pullulan by *Aspergillus japonicus-VITSB2*.Therefore, three response surface graphs were obtained considering all possible combinations. The graphical representation helped to visualize the relation between the response and experimental level of each variable. Each contour plot represents an infinitive number of combinations of two test variables while the other was kept at constant value.

The response surface plots obtained are shown in Fig. 7A represents the interaction between sucrose and peptone (AB), at a specific yeast extract concentration and also indicated their effect on pullulan elaboration. This figure indicated that at low sucrose and peptone concentration pullulan production was less and it increases gradually when the concentration of sucrose and peptone is increased in the medium. However, it is also observed that very high concentration of sucrose has a negative effect on pullulan production. Maximum pullulan production in these conditions was found to be 73.63 g/L . This is also significantly higher compared to earlier published report where, 44.4 g/L pullulan was obtained after optimizing a synthetic media containing sucrose as the carbon source [4] .

The interaction between sucrose and yeast extract (AC) when the concentration of peptone was kept constant. This graph also indicated that higher concentration of yeast extract is not favorable for pullulan production (Fig. 7B). The results showed that pullulan production was increased significantly in comparison to the mean observed response. It was also possible to optimize the media components to obtain high concentration of pullulan.

The response for the interactive factors, of peptone and yeast extract (BC), is shown in Fig. 7C when sucrose was kept at a fixed concentration. It is to be noted that at lower concentration of peptone and yeast extract the pullulan production was less but increases with increase in concentration of those two media components. However, the effect of peptone concentration on pullulan production is more significant as compared to yeast extract. The parity plot showed a satisfactory correlation between the actual and

predicted values, wherein, the points clustered around the diagonal line which indicates the good fit of the model (Fig. 7D).

CONCLUSION

Aspergillus japonicus-VIT-SB2, a non-pigmented pullulan producing strain was screened by UV and EMS mutagenesis. Various physical parameters were studied in order to maximize the pullulan production. The results obtained from RSM, indicated that peptone has significant effect as compared to yeast extract in case of pullulan elaboration. Validation experiments were performed and the results obtained showed that experimental values were in agreement with predicted values. The pullulan yield of 73.63 g/L is also significantly higher as compared to earlier published reports. This may lead to the development of a cost-effective process for pullulan production.

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