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Response surface optimization of fermentation parameters for pullulan elaboration from jaggery by *Aureobasidium pullulans* MTCC 2195

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ABSTRACT

Central composite design of response surface methodology was applied to understand the interactions between the process parameters for pullulan production by *Aureobasidium pullulans* MTCC 2195 using a second order quadratic model. A total of 20 shake-flask runs were conducted and response (both 3 dimensional surface and contour) plots were generated to study the interaction. From analysis of variance (ANOVA) results, it was observed that total fermentation time (h) plays a critical role and has shown significant influence on pullulan production compared to initial pH of medium. No previous work has used statistical optimization of process parameters in pullulan biopolymer production from jaggery. A maximum pullulan concentration of 15.589 g/l was obtained at the optimum levels of parameters (initial jaggery concentration 50 g/l, initial pH 5.0, total fermentation time 120 h).

KEY WORDS: Pullulan, RSM, CCD, Jaggery, Optimization.

1. INTRODUCTION

Pullulan, an exo-, homo-, water soluble carbohydrate biopolymer composed of maltotriose repeating subunits and has linear interconnections of α-(1→4) and α-(1→6) coupled linkages in the ratio of 2:1. Owing to its distinctive structural and discrete physical properties, it provides structural flexibility, easy derivatibility and superior solubility. Pullulan also possess spectacular physico-chemical properties like low oxygen permeability, film and fiber forming capacity, biodegradability, biocompatibility, etc. All these special features have made this fungal exopolysaccharide, as a potential candidate for many of its application in food, pharmaceutical, cosmetic and biomedical fields (Singh, 2008; Oguzhan, 2013).

In defiance of these potential applications, pullulan attracts high price ($ 25 Kg^{-1}) compared to other exopolysaccharides, limits its cost-effective application (Tibaul, 2007). Its high price can be attributed to several reasons like low pullulan yield, high viscous fermentation broths, and costlier raw materials. These could be overcome by studying the pattern of interaction among fermentation conditions and supplementing cheaper carbon substrates in the medium. For this purpose, this study uses an agro-industrial product called jaggery as limiting carbon substrate, for successful production of pullulan. Further, classical one-point optimization techniques are cumbersome, time consuming and overlook the interaction among variables, and therefore, can lead to misinterpretation of results (Chi, 2003; Francis, 2003).

Response surface methodology (RSM) is a statistical deign used as a modeling and optimization technique for identifying effective factors and interaction among factors, which have significant influence on the final yield of product, in many bioprocesses (Oskouei, 2008; Smith, 1997). Central composite design (CCD) of RSM helps in estimating the key factors out of large number of media components and fermentation conditions by performing low experimental runs (Prakash, 2005). Analysis of variance (ANOVA) of this design helps to determine the coefficients of quadratic model and has been extensively used in bioprocess optimization (Li, 2002; Urkut, 2007).

Recently several researchers (Jiang, 2010; Goksungur, 2011; Choudhury, 2012; Sharmila, 2013; Srikanth, 2014; Padmanaban, 2015), have successfully applied RSM technique for medium constituents and process conditions using different strains of *Aureobasidium pullulans* and wide variety of natural carbon substrates. In the present study, RSM has been attempted to examine the interactions among initial jaggery concentration, initial pH and total fermentation time for pullulan elaboration by *A. pullulans* MTCC 2195 in jaggery based shake-flask fermentations. To best of our knowledge, this is the first ever report on the statistical analysis using CCD to optimize the conditions for pullulan production on jaggery medium.

2. MATERIALS AND METHODS

Microorganism and Chemicals: *Aureobasidium pullulans* MTCC 2195, a melanin-free strain, was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. It was maintained at 4^0C on potato dextrose agar (PDA) and sub cultured every fortnight. Biochemicals (of analytical grade) were purchased from M/s. HiMedia Laboratories Pvt. Ltd., Mumbai, India and synthetic chemicals were purchased from M/s. Qualigens fine chemicals, India, M/s. Merck India Ltd., Mumbai, India.

Inoculum and media preparation: Inoculum was prepared by transferring a loopful of stock culture to growth medium (g/l) consists of sucrose 50, yeast extract 3.0, KH_2PO_4 5.0, $MgSO_4.7H_2O$ 0.2, KCl 0.6 and NaCl 1.0. The cultivation was conducted at 30^0C for 48 h. Jaggery was purchased from local market in Guntur, India and was used in shake-flask fermentation studies.

Shake-flask fermentation: A 5% (v/v) of *Aureobasidium pullulans* as inoculum was inoculated into production media containing varied concentrations of jaggery (25, 50, and 75 g/l), with variation in initial pH (3.0, 5.0, and 7.0), according to the experimental design and other constituents same as in growth media. In all cases, each 250 ml Erlenmeyer flask containing 50 ml of production media was inoculated and incubated at 30^0C in an orbital shaker with 150 rpm for different times (96, 120, and 144 h).

Determination of biomass and residual sugar: The concentrations of biomass and residual sugar were estimated by drawing samples from fermentation broth at different total fermentation times, as per experimental design, and processed as per Rama Krishna (2016).

Recovery and purification of pullulan: Pullulan content was recovered, at total fermentation times (96, 120, and 144 h), as per experimental design, according to earlier reported methods (Rama Krishna, 2016).

Experimental design and optimization using RSM: Response surface methodology (RSM), is a useful experimental design strategy for selecting optimum conditions for a multivariable system, as it studies the interaction among variables with less experimentation, time and cost. The central composite design (CCD) with 3 factors and 3 levels, including 6 replicates, all the center point has used for a second order response surface. The central composite design was developed as an imbedded factorial matrix with center points and star points (replicate of axial point) around the center point which allows estimation of the curvature.

A full factorial design, which has a factorial point i.e., distance from center of design space and a star point, α, is the distance of center to design space has a value of 1.0, with 3 factors. Each factor was studied into three different levels (-1: Low, 0: Middle, +1: High) and actual design showed a set of 20 experiments which include 6 center points and 14 non-center points (6 axial points and 8 factorial points) (Table.1). The statistical analysis of the data was performed using Design Expert ver.10.0.2.0 (State-Ease, Inc.).

3. RESULTS AND DISCUSSION

Central Composite Design (CCD) is a very useful to determine the optimal level of fermentation conditions and their interaction. From our earlier studies, it was noted that the initial jaggery concentration and initial pH have shown significant effect on pullulan production. So in this study, we performed a total of 15 experiments with varied combinations of initial jaggery concentration (g/l), initial pH and total fermentation time (h) for pullulan production, as per Table 1. The mean observed results of CCD experiments along with predicted response was compared (Table 1). Fisher's statistical test for analysis of variance (ANOVA) was done to perform statistical analysis. ANOVA resulted a quadratic model that suits the data and the model yielded 9 degrees of freedom with 5 degrees of freedom for lack of fit, 5 degrees of freedom for pure error. ANOVA results indicated that model was significant ("Prob > F" less than 0.0001) for desired response i.e., pullulan elaboration and confirmed the more accuracy of model (data not shown).

Second-order polynomial equation for response (pullulan production), Y in terms of coded factors was expressed as follows:

$$Y = +15.59 +0.62A +0.036B +0.86C -0.081AB -0.024AC +0.024BC-1.89A^2 -0.51B^2 -1.8C^2$$

Where A, B, and C are initial jaggery concentration (g/l), initial pH, and total fermentation time (h), respectively. The factors of equation showed significant negative quadratic effects on pullulan production indicates that pullulan concentration increases as the level of these parameters increased above certain values. The high F-value of model i.e., 366.84 implied that the model is highly significant and only 0.01% chance that this model F-value is large could occur due to noise. The percentage coefficient of variation (*CV%*), a measure of degree of precision with which experiments were compared, was obtained as 1.14 indicates that a lower *CV* shows greater reliability of performed experiments (data not shown).

The statistical significance of quadratic model can be checked by its determination coefficient, R^2. Generally, R^2 values varies between 0 and 1 and its value greater than 0.75 and closer to 1, indicates aptness (strength) of model (Haaland, 1989). In this study, the regression square coefficient (R^2) obtained was 0.997 shows that our model can explain 99.7% variation in the response. Moreover, the adjusted R^2 and predicted R^2 values for the model were 0.9943 and 0.9729, respectively, also confirmed the model is expected to predict the response (as the difference is less than 0.2) more accurately in the present case. Further, signal to noise ratio of 53.214 (> 4 is mostly desirable) indicates an adequate signal and thus model can be used to navigate the design space.

The optimum of location, as obtained by differentiation of the quadratic model, for yielding maximal pullulan concentration was with A = 50 g/l, B = 5.0, and C = 120 h. Model predicted the optimal pullulan production corresponding to these values was 15.589 g/l, whereas mean observed value was 15.6 g/l from experiments. Goodness of fit for maximal pullulan concentration was also confirmed by conducting additional triplicate of experiments, which yielded an average maximum pullulan concentration of 15.54 g/l, from these experiments. The *pareto* plot obtained in diagnostics, showed more satisfactory correlation between the experimental and predicted values, wherein, more points clustered on and around the diagonal line, validate the good fit of the model (Fig.1 (a)).

The 3D response surface plots show the graphical representations of regression equation by analyzing the interaction between all the three factors with all possible combinations and also visualizes the relation between the response and experimental level of each variable to estimate the optimum level of each factor required for maximum production of pullulan by *Aureobasidium pullulans* MTCC 2195. Figure 1 (b), (c), (d) portray the interactions between each pair (*AB*, *AC*, and *BC*) of factor at a specific factor (*C*, *B*, and *A*) and their effect, respectively, on pullulan elaboration. Fig. 1 (b) showed that strong interaction between jaggery concentration and initial pH and low initial concentration of jaggery yielded low pullulan concentration, whereas it increases gradually when the jaggery concentration is increase in the medium. Initial pH around 5.0 indicated the maximum pullulan concentration of 15.589 g/l. This showed significantly higher value of maximum pullulan concentration compared to previous report where, 9.3 g/l pullulan was obtained after optimizing media containing sweet potato as the carbon substrate using RSM (Padmanaban, 2015).

Table.1. Experimental design used in RSM studies to understand interaction among fermentation conditions for pullulan elaboration

Standard order	Run numbers	A	B	C	Response	
		Initial jaggery conc. (g/l)	Initial pH	Total fermentation Time (h)	Observed Pullulan (g/l)	Predicted Pullulan (g/l)
1	13	25 (-1)	3 (-1)	96 (-1)	9.96 ± 0.14§	9.80
2	10	75 (+1)	3 (-1)	96 (-1)	11.3 ± 0.08	11.25
3	9	25 (-1)	7 (+1)	96 (-1)	9.98 ± 0.04	9.99
4	7	75 (+1)	7 (+1)	96 (-1)	11.19 ± 0.05	11.11
5	11	25 (-1)	3 (-1)	144 (+1)	11.45 ± 0.1	11.52
6	6	75 (+1)	3 (-1)	144 (+1)	12.89 ± 0.08	12.87
7	18	25 (-1)	7 (+1)	144 (+1)	11.76 ± 0.08	11.80
8	14	75 (+1)	7 (+1)	144 (+1)	12.68 ± 0.19	12.83
9	12	25 (-1)	5 (0)	120 (0)	13.04 ± 0.08	13.08
10	2	75 (+1)	5 (0)	120 (0)	14.33 ± 0.05	14.32
11	19	50 (0)	3 (-1)	120 (0)	14.89 ± 0.06	15.05
12	8	50 (0)	7 (+1)	120 (0)	15.24 ± 0.11	15.12
13	1	50 (0)	5 (0)	96 (-1)	12.66 ± 0.12	12.93
14	3	50 (0)	5 (0)	144 (+1)	14.89 ± 0.14	14.65
15	17	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59
16	20	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59
17	15	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59
18	5	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59
19	4	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59
20	16	50 (0)	5 (0)	120 (0)	15.6 ± 0.03	15.59

§Mean ± S.D. from three replicate experiments; Coded values of (*-1*): Low, (*0*): Middle, (*+1*): High in each factor.

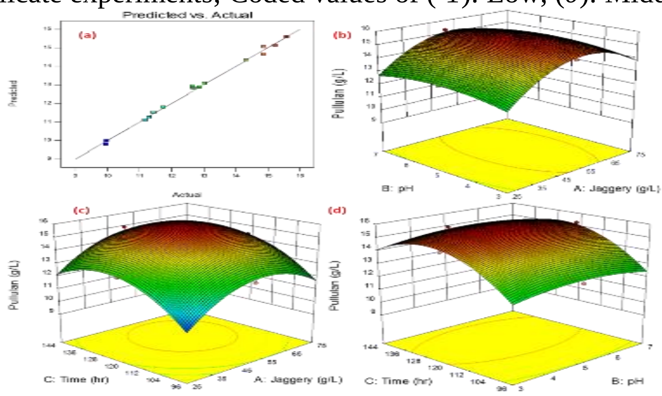

Figure.1. (a) Pareto plot showing the relation between actual and predicted values for pullulan maximization, (b), (c) and (d) 3D Response surface plots for pullulan production using their process variables.

Fig.1 (c) indicates that maximum concentration of pullulan was yielded at the middle level of the variables as contour lines are circular in shape. The mutual effect of initial jaggery concentration and total fermentation time and as total fermentation time is increased, the responses were maximal nearly at the middle of initial jaggery concentration. In earlier reports, optimal conditions for pullulan production were obtained at initial hydrolyzed potato starch waste (as substrate) concentration and fermentation time of 79.4 g/l and 111.84 h, respectively. In Fig.1 (d), the response for the interactive factors, of initial pH and total fermentation time (h), is shown when initial jaggery concentration was kept at a fixed concentration. It is to note that there was a strong interaction existed between the factors pH and time in optimal production of pullulan as the contour is elliptical. Pullulan concentration was reduced at low and high level and increased towards the middle level of pH and time. Hence, the response surface optimization supported 15.589 g/l production of pullulan by *A. pullulans* MTCC 2195 using jaggery as substrate.

4. CONCLUSIONS

Statistical design using RSM can be very appropriate and useful tool to analyze the interactions among fermentation conditions and their profound effect on the yield of pullulan production. In the present study, central composite design (CCD) of RSM has been successfully attempted to study the combined effects of fermentation conditions, such as initial jaggery concentration, initial pH and total fermentation time on shake-flask fermentations of *A. pullulans* MTCC 2195. The results obtained showed that initial pH has most significant effect as compared to total fermentation time in pullulan elaboration. The optimum conditions for fermentation were as follows: initial jaggery concentration, 50 g/l; initial pH, 5.0 and total fermentation time, 120 h. Validation experiments were conducted and the results obtained confirmed that mean observed values were in good agreement with the model predicted values. Thus, use of jaggery may be as an alternative carbon source for cost-effective production of pullulan biopolymer.

5. ACKNOWLEDGEMENTS

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