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Original Research Paper

Statistical optimization of molasses based exopolysaccharide and biomass production by *Aureobasidium pullulans* MTCC 2195

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ABSTRACT

In the present study, optimization of exopolysaccharide (pullulan) and biomass production by *Aureobasidium pullulans* was carried out by four factor—five level central composite design (CCD) of response surface methodology (RSM). Four factors namely molasses, KH_2PO_4, yeast extract and pH were chosen for the optimization studies and their significance on exopolysaccharide and biomass production was statistically analyzed by ANOVA. A second order polynomial model for exopolysaccharide and biomass production was constructed by using the estimated regression coefficients. Optimized values of molasses, KH_2PO_4, yeast extract and pH for targeted response of pullulan (45 g/L) and biomass (12.5 g/L) were predicted as 5.0%, 0.22%, 0.25% and 6.4%, respectively. Result of this study shows that utilization of molasses has significantly improved the exopolysaccharide production and also make the process cost effective.

1. Introduction

Currently, the environmental pollution is the major challenging problem in worldwide. Our environment is mainly polluted by the industrial effluent discharges and accumulation of synthetic non-degradable polymeric materials. These polluting agents affect the ecosystem in land, waterways and also atmosphere by burning the synthetic wastes (Luckachan and Pillai, 2011). Presently, the use of biodegradable polymers for several purposes is significantly increased to reduce the environmental risk. Biopolymers are derived from various sources like plants, microorganisms, animals and can be easily degraded in the environment. This advantage makes the biopolymers more popular than synthetic one. Microbial based biopolymers have gained more industrial importance because of easy scale up and several industrial applications (Nagane et al., 2009; Gounga et al., 2008; Yuen, 1974). Biopolymers such as xanthan gum (Kalogiannis et al., 2003), gellan gum (Banik et al., 2007), pullulan (Sharmila et al., 2013; Gao et al., 2010) and polyglutamic acid (Zhang et al., 2012) by fermentation route were reported in the literature.

Pullulan is a white, non hygroscopic and water soluble biopolymer produced extracellularly by yeast like fungi *Aureobasidium pullulans* (Leathers, 2003). It is composed of maltotriose subunits connected by α-1,6 glycosidic linkages. It is less viscous in nature and shows high stability towards pH and temperature. It is used as filler in beverages, stabilizer and thickener in food industries, binder and tablet coating agents in pharmaceutical industries (Goksungur et al., 2011).

Nutrient components of the production medium mainly affect the yield, product quality and also process cost (Kennedy and Krouse, 1999). Researchers are involved to search and identify a cheap carbon sources for cost effective fermentation process. Utilization of several agricultural based products and byproducts such as jack fruit seed, beet molasses, carob pod, sweet potato and brewery wastes are reported in the literature for pullulan production by fermentative process (Sharmila et al., 2013; Goksungur et al., 2004; Roukas and Biliaderis, 1995; Wu et al., 2009; Roukas, 1999). In this study, molasses is utilized as a low cost substrate for production of pullulan. Molasses is brown viscous liquor waste generated from the sugar industry which consists of fermentable sugars (48–60%) that includes glucose and fructose, organic content (9–12%), inorganic ash (10–15), solids (70–85%) and water (15–30%) (Soni, 2007). It is utilized as a low cost substrate for the production of different bioproducts like ethanol (Bouallagui et al., 2013), biopolymer (Gouda et al., 2001), enzymes (Vohra and Satyanarayana, 2004), pigments (Goksungur et al., 2002) and organic acids etc. (Chan et al., 2012; Jiang et al., 2009). Process optimization is an important step to minimize the cost of any industrial operation. RSM is well accepted tool for optimization

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Table 1
Experimental range and variable levels for RSM optimization.

Factors	Symbol	Lower (−2)	Low (−1)	Middle (0)	High (+1)	Higher (+2)
Molasses, % (v/v)	X_1	1	2	3	4	5
KH_2PO_4, % (w/v)	X_2	0.2	0.3	0.4	0.5	0.5
Yeast extract,%(w/v)	X_3	0.05	0.1	0.15	0.2	0.25
pH	X_4	5.5	6	6.5	7	7.5

studies because of possessing advantage to study the interaction between the variables with less experiments, time and cost (Goksungur et al., 2011; Yu et al., 2008).

The main objective of this study is to optimize the molasses based medium components for the maximized production of exopolysaccharide (pullulan) and biomass by employing RSM. The interaction effect between the responses and the medium variables is also studied.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Fungi culture *A. pullulans* (MTCC 2195) was used in this study. It was procured from MTCC, Institute of Microbial Technology, Chandigarh, India. It was maintained in PDA (Potato Dextrose Agar) slants at 4 °C and sub cultured at every 15 days interval. A loopful of well grown culture was transferred to 100 mL of the inoculum media which consists of glucose 30 g/L, peptone 5 g/L, yeast extract 2.5 g/L, dihydrogen potassium phosphate 1 g/L, magnesium sulphate 0.5 g/L and pH was adjusted to 6.5.

2.2. Preparation of molasses medium

Molasses was obtained from the local sugar industry near Chennai, Tamilnadu. The raw molasses was pretreated with sulphuric acid to eliminate the heavy metals and other undesired compounds (Goksungur et al., 2004). In brief, molasses was acidified to pH 4 with 2 N H_2SO_4 and boiled for 15 min in the water bath. After boiling, the molasses solution was centrifuged at $6000 \times g$ to remove the precipitate and the supernatant was adjusted to pH 6.5 using 2 N NaOH. The pretreated molasses was suitably diluted with distilled water to obtain the final desired sugar concentration for 100 mL and it was used in further studies. The molasses medium consists of molasses 40 g/L, peptone 5 g/L, yeast extract 2.5 g/L, dihydrogen potassium phosphate 1 g/L, magnesium sulphate 0.5 g/L and pH 6.5.

2.3. Fermentation conditions

One hundred milliliters of molasses medium was prepared in 500 mL Erlenmeyer flask and autoclaved at 121 °C. Five percent inoculum was used to inoculate the flask and kept in temperature controlled rotary shaker at 150 rpm, 35 °C for five days. For statistical optimization studies, 100 mL of production medium was prepared in 250 mL Erlenmeyer flask according to the experimental design given in Table 2.

2.4. Biomass estimation

Five milliliters of the culture broth was centrifuged at $11{,}000 \times g$ for 10 min and the supernatant was preserved for the biopolymer extraction. The collected biomass pellet was dried in a hot air oven at 80 °C to a constant weight (Singh et al., 2009).

Table 2
RSM experimental design (in actual units) for production of exopolysaccharide (pullulan) and biomass with observed and predicted responses.

Run order	X_1	X_2	X_3	X_4	Pullulan (g/L)		Biomass(g/L)	
					Observed	Predicted	Observed	Predicted
1	2	0.3	0.1	6	25.4	25.7	8.7	8.8
2	4	0.3	0.1	6	16.8	16.1	9.7	9.6
3	2	0.5	0.1	6	39.8	38.7	8.5	8.5
4	4	0.5	0.1	6	33.4	31.9	8.8	8.9
5	2	0.3	0.2	6	34.3	36.3	9.8	9.7
6	4	0.3	0.2	6	26.6	27.1	10.4	10.5
7	2	0.5	0.2	6	31.5	29.2	9.5	9.7
8	4	0.5	0.2	6	24.1	22.8	10.2	10.1
9	2	0.3	0.1	7	13.2	13.7	7.6	7.9
10	4	0.3	0.1	7	11.8	15.1	9.7	9.4
11	2	0.5	0.1	7	21.0	21.5	8.8	8.6
12	4	0.5	0.1	7	28.5	25.7	9.5	9.7
13	2	0.3	0.2	7	24.6	27.1	8.1	7.8
14	4	0.3	0.2	7	28.5	28.9	9.2	9.3
15	2	0.5	0.2	7	14.9	14.8	8.5	8.8
16	4	0.5	0.2	7	18.7	19.4	10.2	9.9
17	1	0.4	0.15	6.5	32.5	31.4	9.2	9.0
18	5	0.4	0.15	6.5	25.5	26.4	10.7	10.9
19	3	0.2	0.15	6.5	29.9	25.7	8.6	8.7
20	3	0.6	0.15	6.5	25.1	29.2	9.0	9.0
21	3	0.4	0.05	6.5	15.1	16.0	8.9	8.9
22	3	0.4	0.25	6.5	21.5	20.4	9.9	10.0
23	3	0.4	0.15	5.5	29.4	31.6	9.2	9.1
24	3	0.4	0.15	7.5	18.6	16.2	7.9	8.0
25	3	0.4	0.15	6.5	17.9	20.5	8.5	8.6
26	3	0.4	0.15	6.5	19.2	20.5	8.9	8.6
27	3	0.4	0.15	6.5	23.2	20.5	8.7	8.6
28	3	0.4	0.15	6.5	21.9	20.5	9.1	8.6
29	3	0.4	0.15	6.5	20.5	20.5	8.4	8.6
30	3	0.4	0.15	6.5	21.2	20.5	8.2	8.6
31	3	0.4	0.15	6.5	19.7	20.5	8.6	8.6

2.5. Extraction of pullulan

Pullulan levels were determined by precipitating the polysaccharide in the clarified supernatant obtained from previous step with twice the volume of isopropyl alcohol kept at 4 °C for 12 h. Crude biopolymer precipitate was separated by centrifuging the content at $10{,}000 \times g$ for 10 min followed by drying at 80 °C overnight and then weighed (Singh et al., 2009).

2.6. Response surface methodology

Statistical optimization of the pullulan production by RSM was performed by using Minitab software (Myers and Montgomery, 2002). Central composite design (CCD) was used to determine the optimal level of the selected factors. Four factors (Molasses (%), KH_2PO_4 (%), yeast extract (%) and pH) were selected for the optimization at five levels. The experimental range and design were given in Tables 1 and 2, respectively. Experiments were carried out as per the combination of the selected factors and the responses (pullulan concentration and biomass concentration) were tabulated in Table 2. A second order polynomial model shown in Eq. (1) was used to represent the relation exists between the dependent and independent variables.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_{12} + b_{13}X_{13} + b_{14}X_{14} + b_{23}X_{23} + b_{24}X_{24} + b_{34}X_{34} \quad (1)$$

where Y is a response, b_0 is constant, b_1, b_2, b_3, b_4 are linear effect coefficients, b_{11}, b_{22}, b_{33}, b_{44} are quadratic effect coefficients, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} are interaction effect coefficients and X_1, X_2, X_3, X_4 are the independent variables. The obtained data were