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# Cost effective production of pullulan from agri-industrial residues using response surface methodology

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ABSTRACT

Response surface methodology was used to develop an economically feasible process for the fermentative production of pullulan using agri-industrial residues, jaggery, de-oiled jatropha seed cake (DOJSC) and corn steep liquor (CSL), as sole media components. A second order polynomial model was obtained using central composite design to understand the effects of interactions among these substrates on pullulan biosynthesis. Results indicated that, lower concentrations of CSL and DOJSC and higher concentrations of jaggery favoured pullulan production. The optimal nutrient composition (18% jaggery, 3% DOJSC and 0.97% CSL) as suggested by the model resulted in production of 66.25 g/L pullulan with a productivity of 0.92 g/L h. Analysis of raw material cost component for pullulan production suggested that sole utilization of agri-residues may lead to development of cost effective process for pullulan production.



## 1. Introduction

Pullulan is a water soluble neutral extracellular homopolysaccharide synthesized by yeast like fungus *Aureobasidium pullulans*. This biomacromolecule is composed of maltotriose subunits connected by $\alpha$-(1→4) and $\alpha$-(1→6) glycosidic linkages in a unique 2:1 ratio. This unique linkage pattern confers pullulan higher solubility and structural flexibility and renders it special physicochemical properties like fibre and film forming capability, low oxygen permeability, higher mechanical strength, etc. These properties coupled with non-toxic and non-immunogenic nature makes pullulan an ideal choice for applications in food, pharmaceuticals, cosmetic, biomedical industries, etc. Despite these advantages pullulan is less exploited due to its high cost compared to other biopolymers [1]. The high cost of pullulan is associated with cost of raw materials, low productivity and yield during fermentative production and purification of the final product. One of the problems associated with productivity and yield is inability of the *A. pullulans* strains to tolerate higher concentrations of glucose or other carbon sources and this may be overcome by using osmotolerant strains of *A. pullulans* [2]. Attempts were made to increase the fermentative yield and productivity by examining the effect of media composition, pH, temperature, metal ion concentrations, etc. [3–9]. Single point and statistical techniques were also used to enhance the pullulan production by optimizing the media composition and process parameters [10–17]. However, in all these cases, expensive conventional media components like dextrose, yeast extract, peptone, etc. were used and replacing these costly nutrients by low cost substrates may be economically advantageous. Media containing a combination of conventional nutrients along with agri-industrial residues were also used as substrates for fermentative production of pullulan [18–23]. However, there are no reports of using only agri-industrial residues as substrates for production of pullulan. In the present study, a medium solely composed of agri-industrial residues viz. jaggery, de-oiled jatropha seed cake (DOJSC) and corn steep liquor (CSL) was used for production of pullulan using an osmotolerant strain of *A. pullulans*. RSM was applied to develop a model using central composite design to understand the interactions among these nutrients and their effect on pullulan production. The results indicated that pullulan production was favoured at higher concentrations of jaggery and lower concentrations of DOJSC and CSL. Process economic analysis was carried out about the feasibility of using these low cost raw materials for commercial production of pullulan. To the best of our knowledge, this is the first report of pullulan production using a media containing only agri-industrial residues as sole media components.

## 2. Materials and methods

### 2.1. Material

DOJSC was obtained from Biodiesel Production Facility, Centre for Rural Development, Indian Institute of Technology, Delhi, India. A proximate analysis data indicated that DOJSC mostly consists of protein and carbohydrate [24]. CSL was obtained from

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Bharath Starch Industries Limited, Yamunanagar, India. Literature suggested that protein is the major component of this agri-residual by product [25]. Jaggery was procured from the local market and found to be mainly composed of sucrose [26]. The proximate composition of all the media components used are summarized in supplementary Table 1.

### 2.2. Microorganism and inoculum development

*Aureobasidium pullulans* RBF 4A3, isolated from inflorescence of *Caseulia axillaries* [2] was used for pullulan production. The stock culture was stored in 20% glycerol at −80 °C for long term preservation. The organism was sub-cultured in YPD agar plates prior to each experimental run. Freshly grown cultures from YPD plates were transferred to a 250 ml conical flask containing 50 ml of media comprised of 2% (w/v) dextrose, 1% (w/v) yeast extract and 2% (w/v) peptone for inoculum development [27]. The culture was incubated at 28 °C at 200 rpm for 24 h.

### 2.3. Shake flask fermentation

Pullulan was produced in shake flasks using jaggery, DOJSC and CSL as media components. The concentrations of the nutrients in media were varied as suggested by the statistical design of experiments as described earlier [27]. In each case, 20 ml of the production media in a 100 ml conical flask was inoculated with 5%(v/v) inoculum and incubated at 28 °C in a rotary shaker with 250 rpm for 72 h. All the experiments were carried out in triplicates and average data were presented.

### 2.4. Purification, analysis and characterization of pullulan

Pullulan was harvested and purified by following the method as described earlier [2]. Briefly, the fermentation broth was centrifuged at 16,000 × g for 20 min at 4 °C using Sigma 6K-15 centrifuge, followed by filtration through 0.22 μ filters. The polysaccharide was precipitated from this cell free fermentation broth by adding cold absolute ethanol in the ratio of 1:2 (v/v). The precipitate obtained was dried at 80 °C overnight to remove residual ethanol. This crude polysaccharide was further purified using dialysis and re-precipitation techniques as described earlier by Choudhury et al. [2]. Pullulan content in the purified polysaccharide was measured by enzymatic method [22] and was characterized using FT-IR spectroscopy [2].

### 2.5. Design of experiments and statistical analysis

Response surface methodology was used to understand the interactions among nutrients and their effect on production of pullulan. A rotatable central composite design (CCD) was developed as a full factorial matrix to identify the key factors for higher yield of pullulan. In full factorial design α-value gives the distance from centre point to the axial point and also determines the location of axial point in a design space. In case of a full factorial design, the value of $\alpha$ is equal to $(2k)^{1/4}$. In present case the value of $k$ is equal to 3 and hence the value of $\alpha$ is 1.68179. The three media components (jaggery, DOJSC and CSL) were used as numeric factors and varied in 5 different levels ($-1-\alpha$, $-1$, 0, +1, $+1+\alpha$) to generate second order response surface (Table 1). The upper and lower limits of concentrations for all media components were decided on the basis of the results obtained from preliminary experiments. Thus using this full factorial matrix a set of 20 experiments were designed which include 6 centre points, 6 axial points and 8 factorial points with 6 replicates around centre point to ensure proper estimation of the curvature (Table 2). Design Expert software Ver 8.0 (Stat Ease Inc.) was used to perform the regression analysis of the data obtained by performing these experiments and their effect on the selected response (production of pullulan). A second order polynomial equation was developed to define the predicted responses in terms of independent numeric variables and the equation is as follows:

$$Y = x_o + x_1A + x_2B + x_3C + x_{11}A^2 + x_{22}B^2 + x_{33}C^2 + x_{12}AB + x_{13}AC + x_{23}BC.$$

where $Y$ is response, $x_o$ 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 interaction coefficients.

The model developed by regression analysis is a rotatable design and hence may be used to measure and analyze the responses obtained from any combination of the variables within the entire range. The regression model developed was further analyzed statistically after obtaining the responses from the experimental runs performed to determine analysis of variants (ANOVA). Suitability of the model from statistical point of view was evaluated using Fishers test value ($F$ value) and proportion of variance ($R^2$ value) [27]. Design Expert software (ver 8.0) was used to generate contour plots and response surface graphs for each variable. The three dimensional response surface plots were used to understand the individual and combinatorial effect of the interactions among variables on the desired response. These plots were also used to predict optimum media composition for pullulan production. Further the model was validated performing experimental runs suggested during statistical optimization of the model. Experiments were carried out in triplicates and average data were reported.

**Table 1**
Experimental range of the variables studied using CCD in terms of coded and actual factors.

| Factors | Symbols | Coded levels | | | | | |
|---|---|---|---|---|---|---|---|
| | | Min. (%) | Low (−1) (%) | Mid (o) (%) | High (+1) (%) | Max. (%) | Std. Dev. |
| Jaggery | A | 9.95 | 12 | 15 | 18 | 20.05 | 2.48 |
| DOJSC | B | 1.98 | 3 | 4.5 | 6 | 7.02 | 1.24 |
| CSL | C | 0.33 | 0.50 | 0.75 | 1 | 1.17 | 0.21 |

**Table 2**
Experimental design used in RSM studies to understand interaction among nutrients.

| Run no. | Factor1 | Factor2 | Factor3 | Predicted response | Observed response |
|---|---|---|---|---|---|
| | Jaggery (%) | DOJSC (%) | CSL (%) | Pullulan (g/L) | Pullulan (g/L) |
| 1 | 15 | 4.50 | 0.75 | 53.70 | 54.02 |
| 2 | 15 | 4.50 | 0.33 | 56.07 | 54.98 |
| 3 | 18 | 6.00 | 1.00 | 55.82 | 54.50 |
| 4 | 15 | 4.50 | 1.17 | 48.40 | 48.43 |
| 5 | 15 | 4.50 | 0.75 | 53.73 | 54.02 |
| 6 | 18 | 3.00 | 1.00 | 57.61 | 58.59 |
| 7 | 12 | 3.00 | 1.00 | 47.28 | 47.49 |
| 8 | 12 | 3.00 | 0.50 | 51.73 | 53.78 |
| 9 | 18 | 3.00 | 0.50 | 55.82 | 55.89 |
| 10 | 15 | 4.50 | 0.75 | 54.53 | 54.02 |
| 11 | 15 | 4.50 | 0.75 | 53.04 | 54.02 |
| 12 | 15 | 4.50 | 0.75 | 55.80 | 54.02 |
| 13 | 15 | 1.98 | 0.75 | 60.61 | 58.98 |
| 14 | 12 | 6.00 | 1.00 | 40.21 | 40.87 |
| 15 | 15 | 7.02 | 0.75 | 52.93 | 53.50 |
| 16 | 15 | 4.50 | 0.75 | 53.16 | 54.02 |
| 17 | 18 | 6.00 | 0.50 | 55.47 | 55.99 |
| 18 | 9.95 | 4.50 | 0.75 | 43.90 | 42.65 |
| 19 | 12 | 6.00 | 0.50 | 51.60 | 51.35 |
| 20 | 20.05 | 4.50 | 0.75 | 55.69 | 55.88 |