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Statistical optimization of pullulan production from Asian palm kernel and evaluation of its properties

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ABSTRACT

Pullulan, a biodegradable exopolysaccharide, was produced from Asian Palmyra palm kernel by solid-state fermentation. Levels of medium variables, namely carbon to nitrogen ratio (C/N), pH, NaCl concentration and $ZnSO_4{\cdot}5H_2O$ concentration were optimized to maximize pullulan production using Box–Behnken design of experiments. Optimal values were predicted as: C/N ratio – 28.1, pH – 6.6, NaCl – 0.78 g/l and $ZnSO_4{\cdot}5H_2O$ – 0.37 g/l. Theoretical pullulan yield predicted under optimum condition was 30.4 mg/gds. Pullulan was produced under optimum condition and an experimental yield of 28.7 ± 0.3 mg/gds was obtained. ^{13}C NMR spectra of the exopolysaccharide produced from Asian palm kernel revealed the presence of anomeric α (1→6) linked maltosyl units. The weight–average molecular weight of the polymer was determined to be 8.4×10^6 Da by gel permeation chromatography. Thermal decomposition temperature of pullulan was obtained to be 245 °C. The tensile strength of pullulan film (0.5 mm thick) was found to be 27 MPa.

1. Introduction

Pullulan, one of the commercially important exopolysaccharides, is produced by *Aureobasidium pullulans*. The applications of pullulan in pharmaceutical, food and biomedical fields are well recognized [1–10]. In spite of its commercial value, pullulan appears to be economically less competitive owing to its high production cost [11].

Cost of substrate contributes significantly to the pullulan production [11–13]. Previous studies have shown that the cost of production could be cut down by 30% if suitable cost-effective medium is employed in fermentation [11]. In order to reduce the raw material cost, production of pullulan by *Aureobasidium pullulans* has been investigated extensively using various lignocellulosic wastes, in submerged fermentation [10–27]. However, studies on pullulan production by solid-state fermentation are limited.

Solid-state fermentation is superior to submerged fermentation owing to its inherent advantages. It is simple, requires less energy and provides a better yield [26]. In our previous work, we have successfully produced pullulan from a low cost carbon source, Asian Palmyra palm kernel, in solid state fermentation [27].

Asian palm kernel, an agricultural by-product, was selected as a substrate owing to its rich carbohydrate content, low cost and abundant availability [27]. We had reported the influence of medium pH on *A. pullulans* morphology and the yield of pullulan [27].

In this study, the growth medium was optimized using response surface methodology. Response Surface Methodology (RSM) is a useful technique in determining the interaction in a multi-variate process and achieving overall optimization with the requirement of less number of physical experiments and hence, is adopted in our present work. It is well recognized and widely used for process optimization in white biotechnology [17–19,26,28,29].

Various factors like amount of carbon source, concentration of nitrogen sources (like $NaNO_2$ and yeast extract), initial pH, concentration of NaCl, concentration of $MgSO_4{\cdot}7H_2O$ and concentration of $ZnSO_4{\cdot}5H_2O$ were considered. After screening variables that significantly influenced the process, using Plackett–Burman fractional factorial design [results not shown], the levels of the screened variables were optimized to achieve maximum pullulan yield using Box–Behnken design of experiment.

The weight–average molecular weight, thermal stability and mechanical strength, which are important properties of pullulan in industrial application perspective, were evaluated for the product obtained from Asian palm kernel.

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Table 1
Experimental design for pullulan production using Box–Behnken design.

Run order	C/N ratio	pH	NaCl (g/L)	$ZnSO_4 \cdot 5H_2O$ (g/L)	Observed pullulan yield (mg/gds)	Predicted yield (mg/gds)
1	10	2.6	0.6	0.26	9.39	9.40
2	30	2.6	0.6	0.26	17.89	18.08
3	10	6.6	0.6	0.26	20.50	20.40
4	30	6.6	0.6	0.26	28.87	29.08
5	20	4.6	0.1	0.02	13.24	13.12
6	20	4.6	1.1	0.02	19.67	19.53
7	20	4.6	0.1	0.5	18.97	19.26
8	20	4.6	1.1	0.5	21.34	21.62
9	10	4.6	0.6	0.02	12.56	12.54
10	30	4.6	0.6	0.02	21.34	21.22
11	10	4.6	0.6	0.5	15.88	16.66
12	30	4.6	0.6	0.5	24.56	25.34
13	20	2.6	0.1	0.26	11.50	10.99
14	20	6.6	0.1	0.26	20.80	21.99
15	20	2.6	1.1	0.26	14.48	15.38
16	20	6.6	1.1	0.26	26.54	26.38
17	10	4.6	0.1	0.26	13.24	12.91
18	30	4.6	0.1	0.26	20.51	19.99
19	10	4.6	1.1	0.26	16.05	15.70
20	30	4.6	1.1	0.26	26.51	25.97
21	20	2.6	0.6	0.02	12.10	12.44
22	20	6.6	0.6	0.02	21.34	21.40
23	20	2.6	0.6	0.5	15.45	14.52
24	20	6.6	0.6	0.5	28.76	27.56
25	20	4.6	0.6	0.26	24.56	24.89
26	20	4.6	0.6	0.26	24.98	24.89
27	20	4.6	0.6	0.26	25.13	24.89

2. Materials and methods

2.1. Microorganism and culture conditions

A strain of *Aureobasidium pullulans* MTCC 2670 was purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. Stock culture was sustained on potato dextrose agar (PDA) medium at 4 °C and was revived once in every three weeks.

The growth medium comprising the following composition was prepared and sterilized (in g/l): glucose – 50, yeast extract – 2.5, K_2HPO_4 – 5, $(NH_4)_2SO_4$ – 0.6, $MgSO_4 \cdot 7H_2O$ – 0.2 and NaCl – 1. Before sterilization, the pH of the medium was adjusted to 6.8 using 1 N NaOH or 1 N HCl. After cooling, two loops of microorganism were inoculated to 150 ml sterile growth medium. The fermentation medium was then incubated at 30 °C in an orbital shaker for 48 h at 200 rpm.

2.2. Solid state fermentation

Germinated Palmyra palm seeds, after removal of palm sprout, were collected from Sivagangai district, Tamilnadu, India. The outer shell was removed and the kernel was carefully taken and then washed with distilled water to remove the impurities. The kernel was cut into pieces and solar-dried for a week to prevent microbial contamination. The carbon and nitrogen contents of Asian Palmyra palm kernel were determined by CHNS/O analyzer (Series II 2400, Perkin Elmer, USA) and found to be 33.5 wt% and 0.39 wt% respectively.

Asian Palmyra palm kernel (10 g) was added to production medium with 50% moisture content [27] and the mixture was manually agitated for 5 min for uniform wetting. The sample was sterilized, cooled and inoculated with 5% (v/v) seed culture. The fermentation was carried out at room temperature.

2.3. Recovery of biopolymer

After fermentation, 6 volumes of cold distilled water were added to the fermentation sample and uniformly mixed in an orbital shaker for 2 h at 200 rpm [20]. Subsequently, the mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. Solid material with cell debris was carefully gathered and dried in a hot air oven at 90 °C. One volume of supernatant was blended with two volumes of ethanol at 4 °C for 24 h for precipitation. Then precipitated sample was resuspended and centrifuged at 10,000 rpm and 4 °C for 15 min to recover the product. Precipitated sample was dried in hot air oven at 90 °C [23]. The yield of pullulan was expressed in mg of pullulan/g of dry substrate (mg/gds).

2.4. Response surface method

Based on initial screening experiments (results not shown here) the variables such as C/N ratio, pH, and concentration of NaCl (g/l) and $ZnSO_4 \cdot 5H_2O$ (g/l) were chosen for media optimization. Response surface design was carried out to understand the influence of medium/process variables on response variable and their interactions. The design of experiments employed is shown in Table 1. Initial moisture content was adjusted to 50% in all the experiments based on our previously reported study [27].

Both carbon and nitrogen sources play an important role in biopolymer production. Thus, carbon to nitrogen ratio (C/N ratio) is frequently used in media optimization as a single variable rather than two variables (carbon concentration and nitrogen concentration) in fermentation processes [30–37]. The C/N ratio of fermentation medium was varied by the addition of $NaNO_2$ and medium pH was adjusted using citrate phosphate buffer. The yield of pullulan was estimated on the seventh day of fermentation [27]. Totally, 27 experiments were carried out with three center points. The regression analysis was carried out using Minitab 15. A quadratic equation of the following form was used for regression analysis:

$$Y = a_o + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1+1}^{n} a_{ij} x_i x_j \quad (1)$$

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