Research Article

Directive Production of Pullulan by Altering Cheap Source of Carbons and Nitrogen at 5 L Bioreactor Level

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In the present paper, attempts have been made to control the production of pullulan by supplementing commercial source of carbons and protein, timely. Pullulan production was regulated by supplying full fat soya flour and hydrolyzed soya extract, individually and in combination. Pullulan quantification was assayed for sensitivity to pullulanase. *Aureobasidium pullulans* was found to produce 125.7 gL⁻¹ of pullulan. The rotation speed of shake flask, the pH of broth, and the supply of air were maintained at 180 rpm, 5.9, and 1.5 vvm air, respectively. The effect of carbons and lipids on pullulan production was noticed to be substrate specific. However, after the lapse of 36 h, addition of full fat soya flour and hydrolyzed soya extract in combination enhanced the pullulan production 125.7 gL⁻¹. Besides this, pH of broth was also noticed as a critical factor in monitoring pullulan biosynthesis. The newly isolated mutant *Aureobasidium pullulans*, having high potential for pullulan production as compared to existing data, can be well used for commercialization of pullulan.

1. Introduction

Pullulan is an extra cellular water-soluble microbial polysaccharide produced by strains of *Aureobasidium pullulans* [1]. It is a linear mixed linkage α -D-glucan consisting mainly of maltotriose units interconnected via α -(1 → 6) linkages [2, 3]. Typical industrial uses of pullulan are as food coatings and packaging material due to its good film-forming properties, as an ingredient of low calorie foods, and as a starch substitute; as films, with properties similar to those of polyvinyl alcohol, but superior in many ways as well as being biodegradable [4], as an adhesive in the form of pastes with water, as a construction material (after esterification) with fibers similar in strength and elasticity to those in nylon; and as a bulking agent and stabilizer for tablets in the pharmaceutical industry [5, 6]. *A. pullulans* is a polymorphic fungus that synthesizes several polysaccharides, including pullulan [7]. The production of pullulan from a synthetic medium by different strains of *A. pullulans* has been described [8, 9]. The use of agroindustrial wastes like potato starch waste, peat

hydrolyzate, whey, molasses, brewery wastes, and olive oil waste effluents as substrates for pullulan production has also been reported by many researchers. But due to the high cost and much demand of pullulan it has been difficult to widely use this polysaccharide in different type of pharmaceutical formulation and biofilm making [10–12].

2. Methods

2.1. Culture Conditions Optimization for Pullulan Production. *Aureobasidium pullulans* was developed in the author's laboratory by mutation of *Aureobasidium pullulans* MTCC 1991. All the chemicals used were of analytical grade. Lactose, glucose, yeast extract, peptone, sodium chloride (NaCl), ferrous sulphate (FeSO₄), ammonium sulphate (NH₄)₂SO₄, calcium chloride (CaCl₂), and magnesium sulphate (MgSO₄) agar-type I, solvents and reagents used for microbial culture, extraction, separation, product isolation, identification, and purification were obtained from Qualigens (Mumbai, India).

The full fat soya flour, corn flour, wheat flour, and raw molasses were procured from the local market in the batch of March 2011. The acid hydrolysis of full fat soya flour was carried out with H_2SO_4 (25% strength) at 50°C , and concentration of hydrolysed extract was carried out at 90°C in vacuum dryer.

2.2. Shake Flask Level Culture. *Aureobasidium pullulans* culture was maintained on slants and petri plates on a media containing sucrose 40 gL^{-1} , yeast extract 5 gL^{-1} , sodium chloride 5 gL^{-1} and agar 17 gL^{-1} , pH 5.5, and incubated at 28°C . The seed culture media was prepared (glucose 30 gL^{-1} , yeast extract 7.5 gL^{-1} , peptone 5 gL^{-1} , 5.5 pH, sterilized at 121°C , 15 psi for 20 min) and inoculated with single isolated colony, and incubated at 28°C on a temperature controlled orbital shaker (Orbitek, SCIGENICS BIOTECH) at 180 revmin^{-1} for 24 h in a 500 mL Erlenmeyer baffled flask. The 24 h old-seed culture was used as inoculum, 10% inoculum was inoculated in the production medium having the composition (sucrose 50 gL^{-1} , yeast extract 10 gL^{-1} , ammonium sulphate 5 gL^{-1} peptone 2.5 gL^{-1} , FeSO_4 0.2 gL^{-1} , MgSO_4 0.1 gL^{-1} , sodium chloride 5 gL^{-1} , calcium chloride 0.2 gL^{-1}) and incubated at 28°C on a temperature controlled orbital shaker (Orbitek, SCIGENICS BIO TECH) at 180 revmin^{-1} for 168 h in a 500 mL Erlenmeyer baffled flask.

Whereas taking into account for the use of raw cheap carbon sources and nitrogen sources on the yield of pullulan, sucrose was replaced by full fat soya flour, corn flour, wheat flour, and raw molasses (50 gL^{-1}), respectively, in the production media alone or in combination with sucrose. For the cheaper nitrogen source the concentrated hydrolyzed soya extract (15 gL^{-1}) was used alone or in combination with yeast extract/peptone.

2.3. Production of Pullulan at 5 L Fermenter. The 5 L fermenter (B. Braun, Sartorius, Goettingen, Germany) having the working volume of 3.5 L of the production medium was inoculated with 36-h-old 10% seed culture from 500 mL shake flask level. Fermentation was performed at 27°C with 210 revmin^{-1} and 1.25 vvm air for 168 h.

2.4. Determination of Biomass. For determining Dry Cell Weight (DCW), the cells from the broth were obtained by centrifuged at 12000 g for 20 min. The cells were washed two times with N-saline (Sodium Chloride, $0.85\text{ g } 100\text{ mL}^{-1}$) and kept at 80°C for 24 h for drying [12].

2.5. Estimation and Purification of Pullulan. Culture broth (5 mL) was centrifuged at 4000 g for 20 min; 4 mL of supernatant was then mixed with 8 mL of 95% ethanol and incubated at 4°C for 12 h to precipitate the crude products, which were separated by centrifuging at 4000 g for 30 min. The precipitated material was dried at 80°C oven overnight and weighted as the weight of crude pullulan [12]. Polysaccharides produced from *A. pullulans* were assayed for sensitivity to pullulanase to determine the pullulan content [3]. Each polysaccharide sample was first ethanol-precipitated, dried, and subsequently resuspended at a final concentration of

1 mg/mL (0.1%, w/v) in 50 mM sodium acetate buffer (pH 5.0). Pullulanase from *Bacillus acidopullulyticus* (Sigma Aldrich India) was added to a concentration of 0.5 U/mL. After mixing, the mixture was incubated for 24 h at 25°C . Pure pullulan was used as control and data are reported as percentage of reducing sugars relative to complete polysaccharide. Reducing sugar content was determined using DNS method [13]. The calibration curve used for reducing-sugar determination was generated using pure pullulan.

All the experiments were performed in replicates of six at shake flask, and the mean of the experiments was taken as data; while in 5 L jar fermenter, the experiments were performed in triplicate and data collection was the mean of triplicate experiments.

3. Results

The cheap carbon sources tested were full fat soya flour, corn flour, wheat flour, and raw molasses and were used at shake flask level, as an enhancement source for carbon (Table 1). Amusingly, it was noticed that the presence of cheaper in the production media caused does not have any direct impact on biomass production; however the specific production of pullulan yield in full fat soya flour growth media was noticed to be higher, as compared to control. Highest yield of pullulan was observed in shake flask culture having full fat soya flour as carbon source. Full fat soya flour supplied cultures were having significantly higher specific rate of pullulan production compared to control and other cheaper carbon sources.

3.1. Effect of Dissolved Oxygen on Pullulan Fermentation. The effect of pH on growth and production of pullulan at 5 L jar fermenter level shows that pH must be maintained at 5.9. The pH ranging from 5.3 to 6.2 did not show any effect on pullulan production. Though, small change in the pH towards alkaline or acidic condition the specific production of pullulan was significantly decreased (Table 2). The effect of concentrated hydrolyzed soya extract at different concentration at shake flask level on production of pullulan is shown (Table 3). The most effective concentration was found to be 15 gL^{-1} . It was also noticed that by increasing the concentration of concentrated hydrolyzed soya extract above 12 gL^{-1} alone had an adverse effect on pullulan production (Table 3). Interestingly, it was noticed that the addition of yeast extract and peptone along with concentrated hydrolyzed soya extract in 2.0:0.5:12.0 resulted in considerable increase in the production of pullulan (Table 4).

3.2. Effect of Dissolved Oxygen on Pullulan Fermentation. The results in Figure 1 illustrated that the dissolved oxygen had significant role in pullulan fermentation. Furthermore, the rotating rate controlled the level of dissolved oxygen. As from Figure 1, it was noticed that when oxygen was influxed at 1.5 vvm, pullulan production was at the maximum. However, there was a decrease in the production of pullulan with variation in oxygen influx.

TABLE 1: Effect of cheap raw carbon sources on pullulan production.

Different raw carbon 50 gL ⁻¹	DCW in gL ⁻¹	Pullulan production in gL ⁻¹	Specific production g g DCW ⁻¹
Control	27.3 ± 3.11	82.5 ± 3.34	3.04 ± 0.07
Corn flour	25.2 ± 1.22	95.4 ± 4.56	3.80 ± 0.24
Full fat soya flour	29.4 ± 3.25	119.5 ± 3.22	4.10 ± 0.16
Wheat flour	27.5 ± 2.75	79.4 ± 2.43	2.93 ± 0.09
Raw molasses	27.5 ± 2.75	92.5 ± 3.33	3.41 ± 0.11

* DCW-Dry Cell Weight.

TABLE 2: Effect of different pH on pullulan production.

pH	DCW in gL ⁻¹	Pullulan production in gL ⁻¹	Specific production g g DCW ⁻¹
4.6	22.9 ± 0.23	76.4 ± 4.45	3.30 ± 0.03
4.9	23.8 ± 0.31	79.3 ± 3.35	3.43 ± 0.10
5.2	28.6 ± 0.52	115.8 ± 3.08	4.11 ± 0.25
5.5	27.1 ± 31	118.5 ± 1.79	4.37 ± 0.02
5.8	26.3 ± 0.26	115.3 ± 2.45	4.41 ± 0.06
6.2	25.9 ± 0.19	113.1 ± 3.21	4.35 ± 0.12
6.5	23.1 ± 1.1	98.5 ± 2.65	4.21 ± 0.22
6.8	18.3 ± 0.36	75.3 ± 1.35	4.15 ± 0.23
7.2	17.4 ± 0.29	63.1 ± 1.21	3.71 ± 0.17

TABLE 3: Effect of adding concentrated hydrolyzed soya extract in production media on pullulan production.

Concentrated hydrolyzed soya extract gL ⁻¹	DCW in gL ⁻¹	Pullulan productio in gL ⁻¹
Control	27.5 ± 2.01	111.5 ± 2.34
10	28.6 ± 1.78	119.5 ± 1.95
12	29.9 ± 1.50	121.4 ± 1.20
15	30.6 ± 1.75	115.5 ± 1.10
18	30.7 ± 1.62	112.8 ± 1.23
20	31.3 ± 1.45	108.7 ± 1.30

The collective effect of full fat soya flour, glucose, concentrated hydrolyzed soya extract, yeast extract, and peptone was noticed to be interesting. Full fat soya flour, glucose, concentrated hydrolyzed soya extract, yeast extract, and peptone with (50.0 gL⁻¹ : 10.0 gL⁻¹ : 15.0 gL⁻¹ : 2.0 gL⁻¹ : 0.5 gL⁻¹) showed highest quantity of pullulan production (125.7 ± 4.45 gL⁻¹) at pH 5.9 and 180 revmin⁻¹, with aeration at 1.5 vvm.

4. Discussion

Pullulan, a water soluble neutral polysaccharide first reported by Bauer [14], is synthesized intracellularly at the cell wall membrane and secreted out to the cell surface to form a loose, slimy layer [15]. The pullulan precursor, UDPG, is an important medium for pullulan production [16]. The full fat soya provides the lipids and carbon after the completion of the lag phase of growth as described earlier by Pouliot and coworkers [17]. The slow supply of carbon subunits

FIGURE 1: Effect of dissolved oxygen (DO) concentration on bio-mass (DCW = —■—) and pullulan (—▲—) production by *Aureobasidium pullulans* at 5-l-jar fermenter.

and lipids from full fat soya seems to be responsible for the higher production of pullulan as noticed by other workers with other carbon [18] and nitrogen sources [19].

The concentrated hydrolyzed soya extract provides slow essential nitrogen source for the stationary phase cells to be complete in the production phase as it has complex forms of nitrogen compared to the readily available Yeast Extract. The effect of the concentrated hydrolyzed soya extract on production of pullulan was found to be significantly positive as supported in earlier published works [20, 21].

TABLE 4: Effect of different combination of (Yeast extract: peptone: concentrated hydrolyzed soya extract) on pullulan production.

Combination	DCW in gL ⁻¹	Pullulan production in gL ⁻¹	Specific production g g DCW ⁻¹
1 : 1 : 12	28.4 ± 1.45	118.8 ± 2.76	4.15 ± 0.08
2 : 1 : 12	28.2 ± 1.32	121.1 ± 3.21	4.28 ± 0.13
2 : 0.5 : 12	28.3 ± 1.95	125.7 ± 4.45	4.46 ± 0.10
1 : 2 : 12	27.9 ± 1.27	117.5 ± 2.68	4.18 ± 0.09
0.5 : 2 : 12	28.8 ± 1.19	110.3 ± 2.90	3.82 ± 0.11

However, at late lag phase addition of full fat soya and concentrated hydrolyzed soya extract only increased the biomass production but also supported biosynthesis of pullulan, significantly. This could have been due to the use of lipids and complex carbon present in full fat soya and concentrated hydrolyzed soya extract acts as immediate precursor for the biosynthesis of pullulan chains by supplying carbon and lipid compounds.

The addition of concentrated hydrolyzed soya extract appreciably affected the production of pullulan. This could have been due the metabolic support of components, other vitamins, and trace minerals present in concentrated hydrolyzed soya extract which supported the secondary metabolism of *Aureobasidium pullulans* during the stationary phase and in the metabolic pathway of pullulan synthesis [16].

The pH was noticed to be a substantially critical factor in regulating pullulan production as noticed in other microbes used for secondary metabolites production in biopharmaceutical industry [22].

As observed in this study, a shift in pH in a range of ± 1.0 even caused decrease in the production of pullulan significantly. The combined effect of full fat soya flour, sucrose and concentrated hydrolyzed soya extract have a significant positive effect on pullulan production that may lead to the economical industrial production.

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