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Mass Production of Pullulan with Optimized Concentrations of Carbon and Nitrogen Sources by *Aureobasidium pullulans* HP-2001 in a 100-L Bioreactor with the Inner Pressure

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Abstract Cell growth and the production of pullulan by *Aureobasidium pullulan* HP-2001, the UV-induced mutant of *A. pullulans* ATCC 42023, increased with increased concentration of glucose up to 15.0% (w/v). Maximal production of pullulan in the flask scale was 27.65 g/l, when concentrations of glucose and yeast extract were 15.0 and 0.25% (w/v), respectively. Maximal conversion rate of pullulan from glucose as the carbon source was 0.37, when those of glucose and yeast extract were 5.0 and 0.15% (w/v), respectively. On the basis of total amount of pullulan, the conversion rate of pullulan from glucose, and utilization rate of glucose to cell mass and pullulan by *A. pullulans* HP-2001, the optimal concentrations of glucose and yeast extract for the mass production of pullulan were determined to be 10.0 and 0.25% (w/v), respectively, at which concentrations the production of pullulan and its conversion rate were 27.14 g/l and 0.27, respectively. Maximal production of pullulan with optimized concentrations of carbon and nitrogen sources by *A. pullulans* HP-2001 in a 7-l bioreactor was 32.12 g/l for 72 h culture, and that in a 100-l bioreactor with the inner pressure of 0.4 kgf/cm^2 was 36.87 g/l. Increased inner pressure of a 100-l bioreactor resulted in a higher concentration of dissolved oxygen in the medium, which might enhance the production of pullulan by *A. pullulans* HP-2001.

Key words: *Aureobasidium pullulans*, pullulan, carbon source, nitrogen source, optimization, bioreactor, inner pressure

Pullulan is an extracellular, unbranched homo-polysaccharide produced by *Aureobasidium pullulans* [23, 35, 39]. It consists of maltotriose and maltotetrose units with both α-(1→6) and α-(1→4) linkage [5, 6, 8]. Because of its physicochemical properties of its regular alternation of α-1,4 and α-1,6 bonds [20], pullulan produces high-viscosity solutions at relatively low concentrations and can be utilized to form oxygen-impermeable films [28]. Films formed from pullulan are suitable for coating foods and pharmaceuticals, especially when exclusion of oxygen is desirable [45].

Pullulan is one of the few neutral water-soluble microbial polysaccharides that can be produced in large quantities by fermentation [30]. Some important physiological factors that affect the production of pullulan are carbon source [3, 9], nitrogen source [2, 34], temperature [28], initial pH of medium [15, 19, 29], and oxygen supply [24, 42]. One of the undesirable features associated with the production of pullulan by *A. pullulans* is its inhibition through catabolite repression at a high concentration of glucose [40]. Many attempts to eliminate the catabolite repression of glucose have been reported for higher production of pullulan [4, 37, 41].

In this study, *Aureobasidium pullulans* HP-2001, a mutant of *A. pullulans* ATCC 42023, was isolated and it grew in the medium with as much as 20% (w/v) glucose. The production of pullulan by *A. pullulans* HP-2001 was investigated under various concentrations of glucose as a carbon source and yeast extract as a nitrogen source. Furthermore, optimal concentrations of glucose and yeast extract established in the flask scale using batch fermentations of pullulan in a 7-l bioreactor, were performed. The effect of inner pressure in a 100-l bioreactor on cell growth and the production of pullulan by *A. pullulans* HP-2001 were also investigated.

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MATERIALS AND METHODS

Bacterial Strain and Media

Aureobasidium pullulans HP-2001 isolated in this study was the UV-induced mutant of *A. pullulans* ATCC 42023 and produced pigment-free pullulan. It can grow in the medium with as much as 20% (w/v) glucose. *A. pullulans* HP-2001 was transferred monthly to a nutrient agar medium [46]. The medium used for cell growth and the production of pullulan contained the following components (g/l): K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.6; yeast extract (Difco Lab., Detroit. U.S.A.), 2.5 [30]. The pH of the medium was adjusted to 6.0 before sterilization. Glucose was autoclaved separately for 15 min at 121°C and added to the medium under aseptic conditions.

Production and Purification of Pullulan

Starter cultures were prepared by transferring cells from agar slants to 50 ml of medium containing 2% (w/v) glucose in 250-ml Erlenmeyer flasks. The resulting cultures were incubated for 2 days at 30 and 200 rpm. Each starter culture was used as an inoculum for 100 ml of medium in a 500-ml Erlenmeyer flask. Samples were periodically withdrawn from the cultures to examine cell growth and the production of pullulan.

Culture broth after 96 h was centrifuged at 15,000 ×*g* for 15 min to remove cells. Supernatant was mixed with 2 volumes of isopropyl alcohol and incubated at 4°C for 24 h to precipitate crude product which was separated by centrifugation at 15,000 ×*g* for 20 min. The precipitated material was repeatedly washed with acetone and ether, dissolved in deionized water (DW), and dialyzed against DW by using dialysis tubing with a molecular weight cut-off of 14,000 to 12,000 Da. After dialysis for 2 to 3 days with four or five changes of DW, the solution was lyophilized.

Mass Production of Pullulan in 7-L and 100-L Bioreactors

Batch fermentations of pullulan were performed in 7-l and 100-l bioreactors (Ko-Biotech Co., Korea) with working volumes of 5-l and 70-l, respectively. The temperature was maintained at 30°C. Agitation speed and aeration rate of a 7-l bioreactor were 500 rpm and 1.0 vvm, respectively, and those of a 100-l bioreactor were 300 rpm and 1.0 vvm, respectively. The content was agitated by three six-flat-blade impellers in 7-l and 100-l bioreactors, and the inner pressure in a 100-l bioreactor ranged from 0.0 to 0.8 kgf/cm^2. Inoculum size of batch fermentations for the production of pullulan by *A. pullulans* HP-2001 was 5% (v/v).

Analytical Methods

To determine biomass, the cells were washed with distilled water, and dry cell weight (DCW) was measured by directly weighting the biomass after drying to constant weight at 100°C–105°C [18]. The concentration of pullulan was determined colorimetrically by the phenol-sulfuric acid method [10]. A standard curve for quantitation of pullulan was prepared from the authentic pullulan (Sigma, St. Louis, U.S.A.). Reducing sugar contents were determined by the dinitrosalicylic acids (DNS) method [27]. DNS reagent was prepared by first dissolving 7.46 g of 3,5-DNS and 13.98 g of NaOH pellets in 1-l of deionized water. Then, 216.1 g of Rochelle Salt (sodium potassium tartrate tetrahydrate), 5.38 ml of saturated phenol, and 5.85 g of sodium metabisulfite were added, and the reagent was aged for 2 weeks. DNS reagent was added to the same volume of an enzyme-substrate solution, and the preparation was placed in a boiling water bath for 15 min. After the preparation cooled to room temperature, the concentration of reducing sugars was determined at 550 nm with a spectrophotometer (Unicam Co., Helios Delta, U.K.).

RESULTS

Production of Pullulans with Various Concentrations of Glucose

Effect of glucose as a carbon source on cell growth and production of pullulan by *A. pullulans* HP-2001 were investigated (Table 1). With glucose concentration ranged from 0.0 to 20.0% (w/v), cell growth and production of pullulan were found to increase with increased concentration of glucose up to 15.0% (w/v), and the highest production of pullulan was 27.65 g/l when the concentration of glucose was 15% (w/v); however, the conversion rate of pullulan from glucose as a carbon source was relatively low, at 0.18. The highest conversion rate of pullulan and utilization rate of glucose to cell mass pullulan ($Y_{p/s}+Y_{x/s}$) by *A. pullulans* HP-2001 in this study were 0.37 and 0.61, respectively, when the concentration of glucose was 5.0% (w/v). Unlike other strains that showed the catabolite repression against glucose [1, 12, 13, 45], *A. pullulan* HP-2001 grew in the medium with as much as 20% (w/v) glucose and produced pullulan. It seemed likely that *A. pullulan* HP-

Table 1. Cell growth and the production of pullulans by *A. pullulans* HP-2001 with various concentrations of glucose as a carbon source and 0.25% (w/v) yeast extract as a nitrogen source.

Glucose (%)	pH	DCW (%)	Pullulan (g/l)	$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$	Residual sugar (g/l)
0.0	6.70	0.56	0.30	-	-	-	0.00
2.5	5.28	7.10	5.35	0.21	0.28	0.75	2.15
5.0	5.81	11.83	18.50	0.37	0.24	1.52	2.20
7.5	4.76	13.98	26.63	0.36	0.19	1.90	11.91
10.0	4.86	15.35	27.14	0.27	0.15	1.77	18.99
15.0	4.98	17.03	27.65	0.18	0.11	1.62	39.29
20.0	4.88	17.00	26.23	0.13	0.09	1.54	46.02

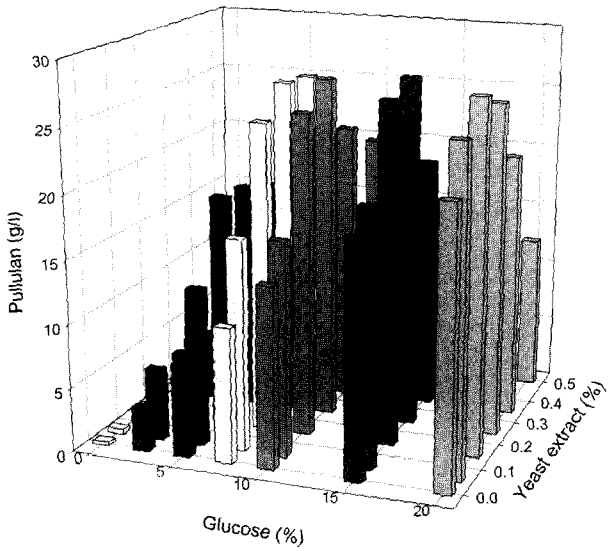

Fig. 1. Production of pullulan by *A. pullulans* HP-2001 with various concentrations of glucose and yeast extract.

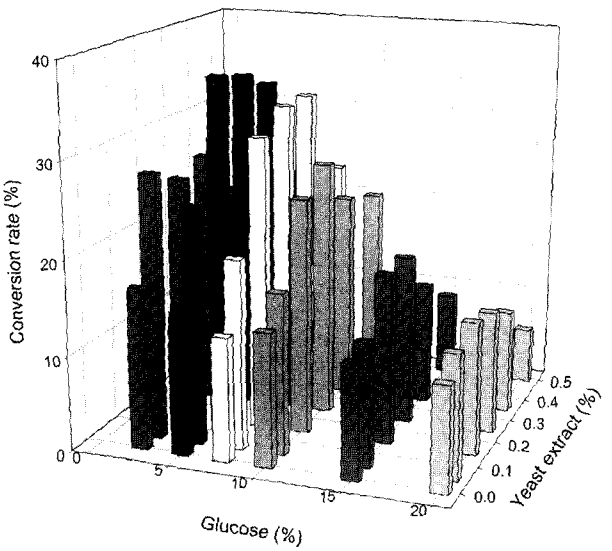

Fig. 2. Conversion rate of pullulan from glucose by *A. pullulans* HP-2001 with various concentrations of glucose and yeast extract.

2001 might have overcome the catabolite repression against glucose.

Optimal Concentrations of Carbon and Nitrogen Sources for the Production of Pullulan

Optimal concentrations of carbon and nitrogen sources for the production of pullulan by *A. pullulans* HP-2001 were examined. Concentrations of glucose as the carbon source and yeast extract as the nitrogen source ranged from 0.0 to 20.0% (w/v) and from 0.0 to 0.5% (w/v), respectively. As shown in Fig. 1, the production of pullulan increased with increased concentration of glucose, and optimal concentration of yeast extract for the production of pullulan varied with concentration of glucose. Maximal production of pullulan was 27.65 g/l, when concentrations of glucose and yeast extract were 15.0 and 0.25% (w/v), respectively, and its utilization rate of glucose to cell mass and pullulan ($Y_{p/s}$+ $Y_{x/s}$) was 0.29. Maximal conversion rate of pullulan from glucose as a carbon source was 0.37, when concentrations of glucose and yeast extract were 5.0 and 0.15% (w/v), respectively (Fig. 2). Cell growth increased with higher concentration of yeast at each concentration of glucose tested in this study, whereas the production of pullulan increased with a limited concentration of yeast (Fig. 3). The nitrogen depletion may be essential for higher production of pullulan, as reported in some other exopolymers [18]. On the basis of total amount of pullulan, conversion rate of pullulan from glucose, and utilization rate of glucose to cell mass and pullulan by *A. pullulans* HP-2001, optimal concentrations of glucose and yeast extract for the mass production of pullulan were determined to be 10.0 and 0.25% (w/v), respectively, at which concentrations the production of pullulan and its conversion rate were 27.14 g/l and 0.27, respectively.

Production of Pullulan with Optimized Concentrations of Carbon and Nitrogen Sources in a 7-L bioreactor

Batch cultures for the production of pullulan by *A. pullulans* HP-2001 were performed in a 7-l bioreactor. In this study, concentrations of glucose and yeast extract in the medium were 10.0 and 0.25% (w/v), respectively, which were predetermined in the flask scale. As shown in Fig. 4, cell growth and the production of pullulan in a 7-l bioreactor gradually increased with culture time. The pH and concentration of the dissolved oxygen in the medium decreased dramatically during the log phase and then

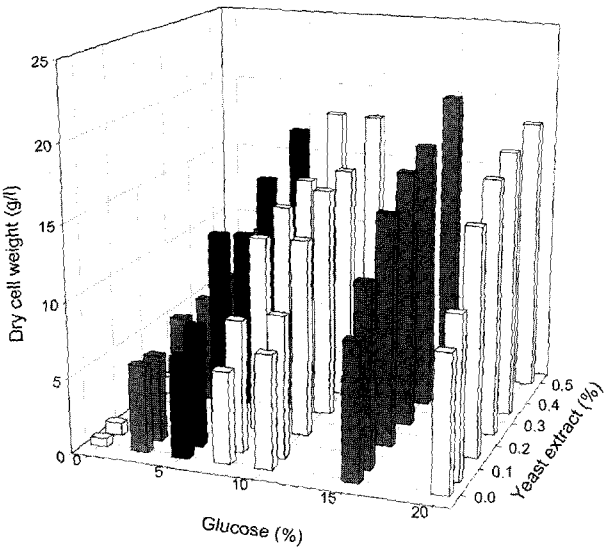

Fig. 3. Cell growth of *A. pullulans* HP-2001, described as dry cell weights with various concentrations of glucose and yeast extract.

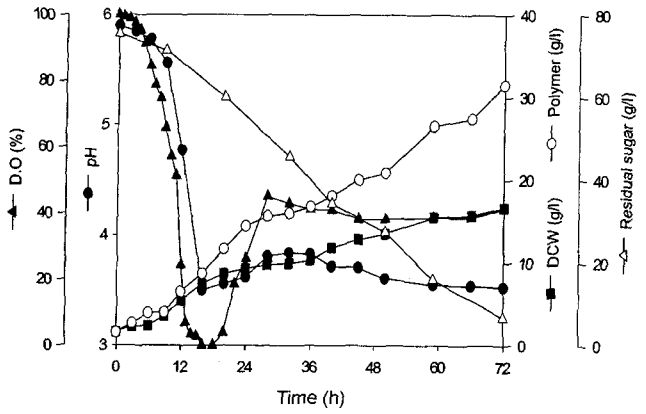

Fig. 4. Cell growth and production of pullulan by *A. pullulans* HP-2001 in a 7-l bioreactor at 30°C with 10% (w/v) glucose, and 0.25% (w/v) yeast extract, at 500 rpm agitation speed, and 1.0 vvm aeration rate.

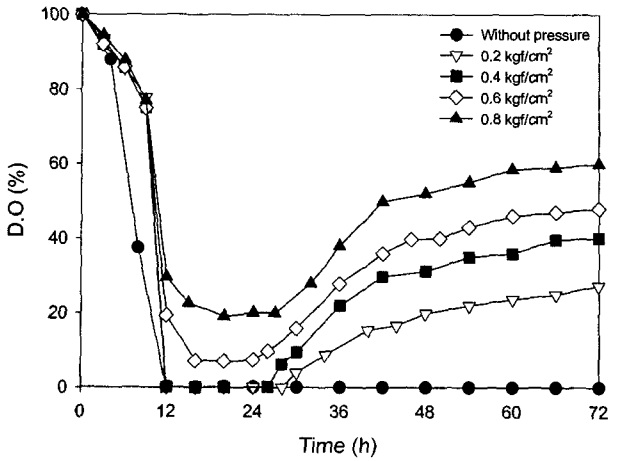

Fig. 5. Concentrations of dissolved oxygen in a medium with various inner pressures in a 100-l bioreactor.

maintained at around 3.2 and 30%, respectively. Reducing sugar in the medium also dramatically decreased with culture time. Maximal production of pullulan by *A. pullulan* HP-2001 in a 7-l bioreactor was 32.12 g/l for a 72 h culture. The molecular mass of the pullulan produced after 72 h is relatively low due to a-amylase secreted into the medium [30]. However, the pullulan with high molecular mass is more useful for applications, such as films, oxygen-impermeable coatings, adhesive, and fibers.

Effect of the Inner pressure on the Production of Pullulan in a 100-L Bioreactor

Effect of the inner pressure on cell growth and the production of pullulan by *A. pullulans* HP-2001 was investigated in a 100-l bioreactor (Table 2). Cell growth increased with increased inner pressure up to 0.8 kgf/cm^2, whereas the maximal production of pullulan occurred at 0.4 kgf/cm^2 in a 100-l bioreactor. As the inner pressure in a 100-l bioreactor increased, the concentration of the dissolved oxygen in the medium increased during culture time, and the time with a shortage of dissolved oxygen in the medium decreased (Fig. 5). Cell growth increased with increased inner pressure in a 100-l bioreactor; however, a limited time with a shortage of dissolved oxygen in the medium seemed to be necessary to enhance the production of pullulan.

Table 2. Effect of the inner pressure of a 100-l bioreactor on cell growth and the production of pullulans by *A. pullulans* HP-2001 with 10% (w/v) glucose and 0.25% (w/v) yeast extract.

Inner pressure (kgf/cm^2)	pH	DCW (g/l)	Pullulan (g/l)	$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0.0	6.70	15.98	28.10	0.28	0.16	1.76
0.2	5.28	16.55	34.86	0.35	0.17	2.11
0.4	5.81	17.14	36.87	0.37	0.17	2.15
0.6	4.76	17.83	34.62	0.35	0.18	1.94
0.8	4.86	18.55	33.74	0.34	0.19	1.82

Batch cultures for the production of pullulan by *A. pullulans* HP-2001 were performed in a 100-l bioreactor with an inner pressure of 0.4 kgf/cm^2 (Fig. 6). Cell growth and the production of pullulan gradually increased with culture time, as shown in a 7-l bioreactor. The pH in the medium decreased and then maintained at around 3.5. The concentration of the dissolved oxygen in the medium dramatically decreased and reached to 0% during the log phase. After a limited time with a shortage of dissolved oxygen in the medium, it increased and maintained at around 40%. Maximal production of pullulan was 36.87 g/l, at which the conversion rate of pullulan from glucose and total utilization rate of glucose to cell mass and pullulan ($Y_{p/s}+Y_{x/s}$) were 0.36 and 0.54, respectively. The production of pullulan with the inner pressure of 0.4 kgf/cm^2 was 1.3 times higher than that without the inner pressure.

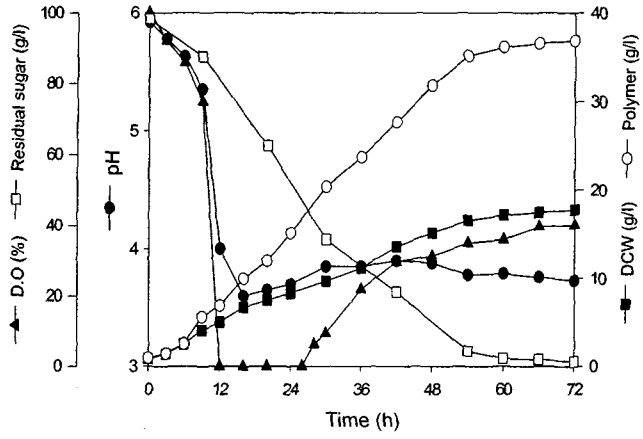

Fig. 6. Cell growth and production of pullulan by *A. pullulans* with 10% (w/v) glucose and 0.25% (w/v) yeast extract in a 100-l bioreactor at 30°C with an agitation speed of 350 rpm, an aeration rate of 1.0 vvm, and an inner pressure of 0.4 kgf/cm^2.

DISCUSSION

One of the most important factors that can enhance the production of pullulan is the concentration of carbon source [33]. The production of pullulan has showed the inhibitory effects of high concentration of glucose [23], implying that a mutant resistant to the catabolite repression against sugars should be obtained to enhance the production of pullulan. The other means to overcome the catabolite repression is to develop new carbon sources other than glucose for the production of pullulan by *A. pullulans*. In order to avoid the catabolite repression against glucose, carbon sources other than glucose including sucrose, corn syrup, starch hydrolysate, and Jaggery, a concentrated sugar cane juice, have been examined [4, 37, 38, 41, 43]. *A. pullulans* HP-2001 grew in the medium with as much as 20% (w/v) glucose in the medium and produced pullulan. In the present study, the highest conversion rate of pullulan from glucose by *A. pullulans* HP-2001 was 0.37 when concentration of glucose was as much as 5.0% (w/v). This is quite dissimilar to other strains, which showed the catabolite repression against glucose to produce exopolymers such as zooglan, heteropolysaccharide-7, and curdlan as well as pullulan [17, 18, 21-23]. The highest conversion rate of the above exopolyemrs occurred at less than 2% (w/v) glucose.

The fungus, *Aureobasidium pullulans*, has a complex life cycle and can grow in various morphological forms including blastospore (yeast-like cells), hyphae, pseudohyphae, swollen cells, and chlamydospores [25]. Also, the productivity of the culture is closely linked to morphological compositions of the culture [7, 13]. The yeast-like cells are the major forms involved in pullulan biosynthesis [14]. High production of pullulan has been found to correlate with high concentration of yeast-like cells in the culture [32]. The morphological content of *A. pullulans* is influenced by process variables, such as carbon and nitrogen sources, pH, temperature, and dissolved oxygen level [26, 42].

In the present study, the effect of inner pressure on cell growth and the production of pullulan by *A. pullulans* HP-2001 were examined in a 100-l bioreactor. Higher concentration of dissolved oxygen in the medium showed the enhancement of cell growth and production of pullulan. However, higher than a certain critical concentration of the dissolved oxygen, which was supplied with increased agitation speeds, aeration rates, or inner pressures, seemed to lead the biosynthetic pathways to produce more intermediates for cell growth, but not for the production of pullulan [42]. Decreased concentration of dissolved oxygen has been suggested to specifically enhance production of glucans in a number of fungi [31, 36]. A limited time with a shortage of dissolved oxygen in the medium seemed to induce the yeast-like morphological type of *A. pullulans* HP-2001 cells, resulting in enhanced production of pullulan.

To the best of our knowledge, this may be the first report on the mass production of pullulan with economical productivity. For industrialization including the optimal inner pressure in a 100-l bioreactor, most of the previous reports on the production of pullulan were performed in flask-scale or in bioreactors smaller than 20-l. Further study will be focused on optimization of physiological conditions for the production of pullulan by *A. pullulans* HP-2001 on the scale of mass production.

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