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Continuous Production of Pullulan by *Aureobasidium pullulans* HP-2001 with Feeding of High Concentration of Sucrose

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Abstract In this study, glucose, sucrose, and dextrin were found to be better carbon sources for the production of pullulan by *Aureobasidium pullulans* HP-2001. Maximal production of pullulan with 200 g/l sucrose as a carbon source was 54.2 g/l. The highest yield of pullulan from sucrose was 0.40, when the sugar concentration was 100 g/l. Optimal conditions for the continuous production of pullulan by *A. pullulans* HP-2001 in a 7-l bioreactor were determined by studying the effects of composition of feed solution, dilution rate, and concentration of sucrose in the feed solution. Pullulan concentration and productivity with 100 g/l glucose and 2.5 g/l yeast extract were 38.1 g/l and 0.53 g/l·h for 72 h, respectively, in a batch culture of *A. pullulans* HP-2001. When the substituted medium contained 100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts, which is the same composition as the medium for the production of pullulan, the pullulan concentration and productivity were 74.9 g/l and 0.55 g/l·h for 120 h, respectively. The production of pullulan at the steady state increased with a dilution rate up to 0.015/h, and its concentration was 78.4 g/l with a weight average molecular weight (M_w) of 4.0×10^5. Unlike a batch culture, however, the decline of the M_w and the number average molecular weight (M_n) of pullulan was not found in the continuous culture of *A. pullulans* HP-2001. When the concentration of sucrose in the feed solution was 200 g/l, 113.5 g/l of pullulan was obtained at the steady state. The steady state was maintained longer in the continuous culture fed with the feed solution containing 200 g/l sucrose than when fed with the feed solutions containing either 100 or 150 g/l sucrose.

Key words: Pullulan, *Aureobasidium pullulans*, continuous culture, dilution rate, molecular weight

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Pullulan is an extracellular and unbranched homopolysaccharide that consists of α-(1→6) linkages of α-(1→4) linked maltotriose units [5, 6, 35, 36]. Pullulan may be used as a coating and packaging material, a sizing agent for paper, a starch replacer in low-calorie food formulation, in cosmetic emulsions, and in other industrial and medicinal applications [8, 39].

Pullulan is one of the few neutral water-soluble microbial polysaccharides that can be produced in large quantities by fermentation [20]. There are, however, several undesirable features associated with the production of pullulan by *A. pullulans* [8, 12]. These include inhibition by high sugar concentration, the decline in molecular weight of pullulan with progression of fermentation and high cost associated with purification of pullulan from the culture broth [30].

Many attempts to eliminate the catabolite repression against glucose have been reported for higher production of pullulan [4, 32, 37]. A mutant of *A. pullulans* ATCC 42023 was previously isolated, and the concentration of glucose as a carbon source and yeast extract as a nitrogen source for the production of pullulan by this strain was optimized [26]. To reduce the cost for mass production of pullulan, soybean pomace as a nitrogen source was developed to substitute yeast extract [27].

For a commercial use, an economical process is required for mass production of pullulan. Continuous culture has also been considered to be a possible method for the higher production of pullulan. Some studies on continuous cultures for the production of exopolysaccharides, such as pullulan [23, 25], xanthan [16], curdlan [19], and alginate [10] have been reported. In this study, we examined sucrose as a carbon source and the effect of medium substitution on cell growth and production of pullulan in a 7-l bioreactor by *A. pullulans* HP-2001. We also examined the optimal dilution rate for the continuous production of pullulan, and the effect

of sucrose concentration in the feed solution on cell growth and production of pullulan.

MATERIALS AND METHODS

Bacterial Strain and Medium

Aureobasidium pullulans HP-2001, a UV-induced mutant of *A. pullulans* ATCC 42023, was transferred monthly to the nutrient agar medium [26]. The medium used for cell growth and exopolymer production 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; and yeast extract, 2.5 (Difco Lab., Detroit. MI, U.S.A.) [36]. The carbon source (20 g/l glucose) was autoclaved separately for 15 min at 121°C and added to the medium under aseptic condition.

Culture Conditions

Starter cultures for shake flasks were prepared by transferring cells of *A. pullulans* HP-2001 from agar slants to 50 ml of medium containing 20 g/l glucose in 250-ml Erlenmeyer flasks. The resulting cultures were incubated for 2 days at 30°C and 200 rpm, and each starter culture was used as an inoculum for 100 ml of medium in 500-ml Erlenmeyer flasks. The inoculum size and culture temperature were 5.0% (v/v) and 30°C, respectively. Samples were periodically withdrawn from the cultures to monitor cell growth and the production of pullulan.

Experiments were carried out with a 7-l bioreactor (Ko-Biotech Co., Korea) with two six-bladed impellers and three baffles, and 4-l working volume. The aeration rate and agitation speed were 1.0 vvm and 500 rpm, respectively. Starter cultures for 7-l bioreactors were prepared by transferring cells from agar slants to 200 ml of medium containing 20 g/l glucose as a carbon source in 500-ml Erlenmeyer flasks. Each starter culture was used as an inoculum for the culture in a 7-l bioreactor. The inoculum size was 5.0% (v/v), and carbon and nitrogen sources for a batch culture were 100 g/l sucrose and 2.5 g/l yeast extract, respectively.

Continuous cultivation was carried out with a varying peristaltic pump speed to feed the feed solution into a 7-l bioreactor. The continuous feeding of feed solution was commenced after 72 h of batch fermentation. Samples were periodically withdrawn from the cultures to monitor cell growth and the production of pullulan.

Purification of Pullulan

Cultured broth was centrifuged at 8,000 ×*g* for 15 min to remove fungal cells. Supernatant was mixed with 2 volumes of isopropyl alcohol and incubated at 4°C for 24 h to precipitate the crude product, which was separated by centrifugation at 8,000 ×*g* for 20 min. The precipitated material was washed repeatedly with acetone and ether, dissolved in deionized water, and dialyzed against deionized water by using dialysis tubing with a cut-off ranging molecular weight from 12,000 to 14,000. After dialysis for 2 days, the solution was lyophilized.

Analytical Methods

To determine biomass, cells were washed with distilled water, and dry cells weight (DCW) was measured by directly weighing the biomass after drying to a constant weight at a temperature of 100°C to 105°C. The concentration of pullulan was determined colorimetrically by the phenol-sulfuric acid method [9]. A standard curve for pullulan was prepared from pullulan (Sigma, St. Louis, U.S.A.). The residual sucrose in the culture broth was determined by the Sucrose Assay Kit (Sigma, St. Louis, U.S.A.), which is used for enzymatic quantitation of sucrose in foods and other materials [34].

Determination of Molecular Weight by GPC

The weight average molecular weight (M_w) (average molecular weight divided by the weight of each polymer chain) and the number average molecular weight (M_n) (average molecular weight divided by the number of molecules) as well as the polydispersity (M_w/M_n) (the breadth of the molecular weight distribution) of the pullulan samples were determined by gel permeation chromatography (Viscotek, U.S.A.) equipped with a TSK PW_{XL} column (Viscotek, U.S.A.) and an RI detector. Pullulan standards with narrow polydispersity and molecular weights, ranging from 5.80×10^3 to 1.60×10^6, were used to construct a calibration curve. Deionized water was used as a mobile phase at a flow rate of 1.0 ml/min. The sample concentration and injection volume were 5.0 mg/ml and 100 μl, respectively. All of the sample solutions were filtered through 0.45-μm-pore-size filters (Adbentec MFS, Inc., Japan) before injection.

RESULTS AND DISCCUSION

Effect of Carbon Sources on Production of Pullulan

The effect of carbon source on cell growth and the production of pullulan by *A. pullulan* HP-2001 was examined (Table 1). Carbon sources used in this study were glucose, gluconic acid, glucosamine, fructose, sucrose, maltose, dextrin, and cellulose at the concentration of 20 g/l. The total utilization yield of substrate for cell growth and the production of pulluan ranged from 0.23 to 0.68. Among the sources tested, glucose, sucrose, and dextrin were found to be better carbon sources for the production of pullulan by *A. pullulans* HP-2001: 6.1 g/l, 6.0 g/l, and 6.2 g/l, respectively, after 72 h with glucose, sucrose, and dextrin.

Effect of Sucrose Concentration on Production of Pullulan

The effect of sucrose concentration on cell growth and the production of pullulan by *A. pullulan* HP-2001 was also

Table 1. Effect of carbon source on cell growth and production of pullulan by *A. pullulan* HP-2001 in a shake flask for 72 h.[a]

Carbon source[b]	pH	DCW[c] (g/l)	Pullulan (g/l)	$Y_{p/s}$[d]	$Y_{x/s}$[e]	$Y_{p/x}$[f]
Glucose	5.4	6.6	6.1	0.31	0.33	0.93
Gluconic acid	8.5	2.5	2.9	0.15	0.12	1.18
Glucosamine	5.8	2.3	1.5	0.07	0.12	0.64
Fructose	6.0	9.4	3.1	0.15	0.47	0.33
Sucrose	5.5	7.6	6.0	0.30	0.38	0.79
Maltose	6.3	9.1	2.7	0.13	0.45	0.30
Dextrin	5.2	7.0	6.2	0.31	0.35	0.87
Cellulose	7.7	2.7	1.8	0.09	0.14	0.66

[a]All data were averages from triplicate experiments.
[b]The concentration of carbon source was 20 g/l.
[c]Dry cells weight.
[d]Yield of product (pullulan) per substrate (sucrose).
[e]Yield of cell mass per substrate.
[f]Yield of product per substrate.

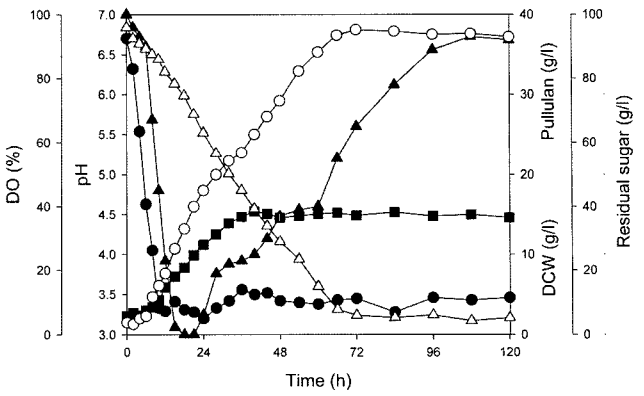

Fig. 1. Cell growth and production of pullulan by *A. pullulans* HP-2001 with 100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts solution in a 7-l bioreactor at 30°C, 500 rpm, and 1.0 vvm (●, pH; ■, dry cells weight; ▲, dissolved oxygen; ○, pullulan; and △, residual sugar).

examined (Table 2). The concentration of sucrose ranged from 0 to 300 g/l, and the production of pullulan increased as the concentration of sucrose as a carbon source increased. The maximal production of pullulan after 72 h was 54.2 g/l, when the concentration of sucrose was 200 g/l. The highest conversion yield of pullulan from sucrose was 0.4, when its concentration was 100 g/l. The total utilization yield of sucrose as a substrate ranged from 0.14 to 0.61, and the highest total utilization yield was found at 20 g/l sucrose. Therefore, *A. pullulans* HP-2001 seems to overcome the catabolite repression by sucrose and utilize a relatively higher concentration of sucrose for the production of pullulan. An excess of carbon source causes catabolite repression. However, in the polysaccharide fermentation, an increase of polysaccharide concentration in a batch culture to utilize high concentration of sugar has advantages for saving the solvent used for the recovery of exopolysaccharides [7, 11].

Production of Pullulan with Sucrose by a Batch Culture

The general growth kinetics of a batch culture of *A. pullulans* HP-2001 with 100 g/l sucrose and 2.5 g/l yeast extract in a 7-l bioreactor is shown in Fig. 1. After 40 h of cultivation, growth of *A. pullulans* HP-2001 reached the stationary phase; however, the production of pullulan continued. The production of pullulan was not concomitant with cell growth. The pH in the medium decreased rapidly and then remained at around 3.5 after the early log phase. The concentration of dissolved oxygen in the medium decreased dramatically and then reached 0% at the middle of the log phase. After a certain period of time with a shortage of dissolved oxygen in the medium, it rose gradually and maintained at around 90% at the final state. A certain period of time with a shortage of dissolved oxygen in the medium has been shown to be necessary to enhance the production of pullulan [26]. The fungus *A. pullulans* has a complex life cycle and can grow in various morphological forms including blastospores (yeast-like cells), hyphae, pseudohyphae, swollen cells, and chlamydospores [17]. High production of pullulan has been found to correlate with high concentration of yeast-like cells in the culture [24], and a certain period of time with a shortage of dissolved oxygen in the culture induces the yeast-like morphological type of *A. pullulans* HP-2001 [26].

Table 2. Effect of sucrose concentration on cell growth and production of pullulan by *A. pullulans* HP-2001 in a shake flask for 72 h.

Concentration (g/l)	pH	DCW (g/l)	Pullulan (g/l)	$Y_{p/s}$	$Y_{x/s}$	$Y_{p/x}$
0	6.8	1.3	0.9	-	-	0.75
20	4.4	6.2	5.9	0.30	0.31	0.95
50	3.4	11.3	17.5	0.35	0.22	1.55
75	3.4	12.5	28.7	0.38	0.17	2.13
100	3.2	13.5	40.0	0.40	0.14	2.96
150	3.2	14.5	47.6	0.32	0.10	3.29
200	3.2	15.1	54.2	0.27	0.08	3.59
300	3.3	19.9	53.0	0.07	0.07	2.67

Maximal production of pullulan was 38.1 g/l, at which the conversion yield of pullulan from glucose and total utilization yield of glucose to cell mass ($Y_{x/s}$) and pullulan ($Y_{p/s}$) were 0.38 and 0.53, respectively. After cultivation for 72 h, the concentration of residual sugar in the culture broth was about 5.0 g/l. Many attempts to eliminate the catabolite repression against carbon sources and reduce the cost for the production of pullulan have been reported with the use of various carbon sources such as peat hydrolysate, molasses, beat molasses, brewery wastes, and agricultural by-products [14, 21, 22, 27]. However, the yields of pullulan from those carbon sources were less than 0.4. The maximum yields of pullulan from starch hydrolysate and potato

starch waste were 34% (17 g/l) and 27% (27 g/l) when concentrations of carbon source were 50 g/l and 100 g/l, respectively [4, 31]. Youssef *et al.* [40] reported a maximum production of 31.3 g/l pullulan in a stirred-tank fermentor when the carbon source was 50 g/l sucrose. The kinetic parameters for the production of pullulan by *A. pullulans* NRRL 6220, which were calculated by applying the Logistic, the Luedking-Piret, and the modified Luedking-Piret equations, showed that an efficient process could possibly be obtained in a fermentation of a shorter duration operated in a sucrose fed-batch mode with 50 g/l sucrose as the starting concentration [18].

Effect of Medium Substitution on Production of Pullulan

The effect of medium substitution on cell growth and production of pullulan by *A. pullulans* HP-2001 in a 7-l bioreactor was examined (Fig. 2). After 72 h of culture, a half of the cultured medium was removed and the same amount of new medium was then added into the bioreactor. Substituted media used in this study were 1) 100 g/l sucrose, 2) 100 g/l sucrose plus 2.5 g/l yeast extract, and 3) 100 g/l sucrose plus 2.5 g/l yeast extract plus mineral salts, which is the same composition as the medium for the production of pullulan. The composition of the mineral salts solution is described in Materials and Methods. The pH of the substituted media was adjusted to 6.0 before sterilization.

When the substituted medium contained only 100 g/l sucrose, the concentration of pullulan and productivity at 120 h were 49.6 g/l and 0.41 g/l·h, respectively (Table 3). The final concentration of pullulan in the medium was similar to that of the batch culture; however, the overall production of pullulan was 1.56 times higher than that by the batch culture without substitution of medium. When the substituted medium contained 100 g/l sucrose and 2.5 g/l yeast extract, the concentration of pullulan and productivity at 120 h were 65.1 g/l and 0.49 g/l·h, respectively. The overall production of pullulan was 1.90 times higher than that by the batch culture. When the substituted medium contained 100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts, the concentration of pullulan and productivity at 120 h were 74.9 g/l and 0.55 g/l·h, respectively. The overall production of pullulan substituted with the fresh medium was 2.10 times higher than that by the batch culture. For fed-batch cultures of *A. pullulans* P 56, the highest values of pullulan concentration (24.5 g/l) and pullulan productivity (0.15 g/l·h) were reported, when grown with feeding substrate containing 50 g/l sucrose and all nutrients including 0.6 g/l yeast extract [40].

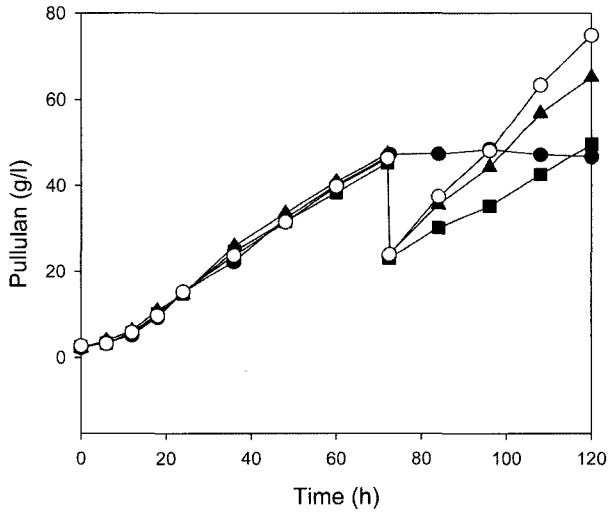

Fig. 2. Effect of medium substitution after 72 h on the production of pullulan by *A. pullulans* HP-2001 (●, batch culture without substitution; ■, substitution of medium containing only 100 g/l sucrose; ▲, substitution of medium containing 100 g/l sucrose and 2.5 g/l yeast extract; and ○, 100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts).

Table 3. Effect of medium substitution after 72 h on the production of pullulan by *A. pullulans* HP-2001 in a 7-l bioreactor.

	Composition of substituted medium			
	N[a]	C[b]	CN[c]	CNM[d]
Culture time (h)	72	120	120	120
pH	3.4	3.4	3.5	3.3
DCW (g/l)[e]	16.7±2.4	14.2±1.5	17.9±2.7	20.5±3.0
Pullulan (g/l)[f]	46.7±3.2	49.6±2.5	65.1±6.5	74.9±5.8
$Y_{p/s}$	0.47	0.49	0.59	0.65
$Y_{x/s}$	0.17	0.15	0.17	0.19
$Y_{p/x}$	2.76	3.27	3.47	3.42
Total amount of pullulan (g)	186.7	291.9	353.9	392.9

[a]No substitution.
[b]100 g/l sucrose.
[c]100 g/l sucrose and 2.5 g/l yeast extract.
[d]100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts solution.
[e]Final dry cells weight.
[f]Final concentration of pullulan.

It is generally believed that pullulan is produced only under N-limitation condition [28]. However, in the present study, a depletion of yeast extract as a nitrogen source in the feed solution was found not to be optimal for the continuous production of pullulan by *A. pullulans* HP-2001. Yeast extract used as a nitrogen source is a complex mixture of amino acids, peptides, and protein, as well as vitamin B and mineral salts [1, 2, 28–30]. It seems that a reasonable amount of yeast extract as a nitrogen source in the feed solution is essential for higher production of pullulan by *A. pullulans* HP-2001.

Effect of Dilution Rate on Production of Pullulan

A. pullulans HP-2001 were continuously cultured in a 7-l bioreactor to investigate the effect of dilution rate on cell growth and the production of pullulan (Fig. 3). Extrapolated

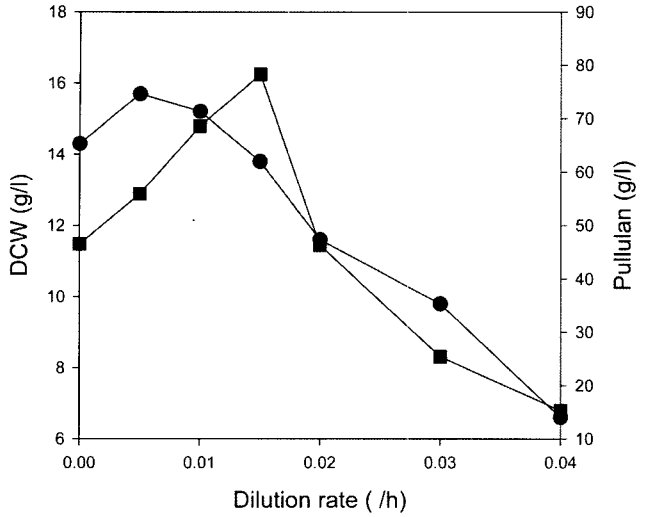

Fig. 3. Effect of dilution rate on the production of pullulan by continuous culture (●, dry cells weight; ■, pullulan).

values for the dilution rate of zero were obtained from a batch culture. The feed solution was the fresh medium containing 100 g/l sucrose, 2.5 g/l yeast extract, and mineral salts. The concentration of pullulan at the steady state increased as the dilution rate was increased up to 0.015/h, and the maximal production of pullulan was 78.4 g/l. Above the dilution rate of 0.015/h, the steady state could not be maintained and a washout of cells occurred. These results indicated that the optimal dilution rate for the production of pullulan was 0.015/h in the continuous culture of *A. pullulans* HP-2001.

The distribution of the molecular weights of pullulan produced by the continuous culture with a dilution rate of 0.015/h is shown in Fig. 4. The weight average molecular weight (M_w) of pullulan increased until the middle of the log phase, and then suddenly decreased until the stationary phase. The highest M_w and the number average molecular weight (M_n) of pullulan after 24 h of culture were 4.20×10^6 and 1.22×10^6, respectively. After feeding of a fresh medium, the M_w and M_n of pullulan increased slightly to around 5.40×10^5 and 1.70×10^5, respectively. The narrowest polydispersity (M_w/M_n) of pullulan was 2.45 at 48 h, and polydispersity increased to around 4.1 with culture time increased. More than 70.0 g/l of pullulan with the M_w of 5.20×10^5 was obtained after feeding of the fresh medium until 264 h.

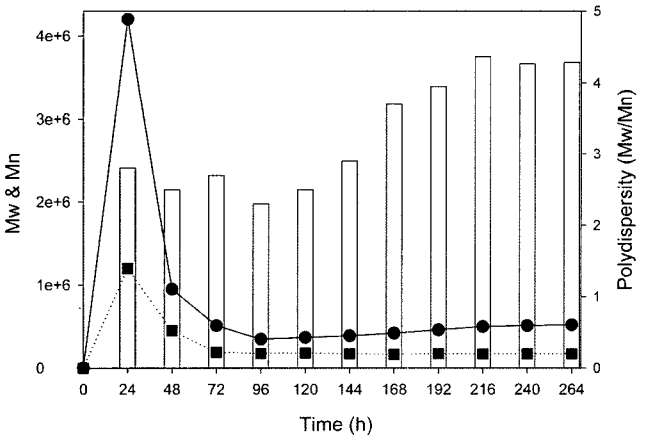

Fig. 4. Distribution of M_w and M_n of pullulan produced by continuous culture with a dilution rate of 0.015/h (●, M_w; ■, M_n; □, polydispersity).

The M_w of pullulan ranged from 1.5×10^4 to 1.0×10^7, depending on culture condition and strains [20, 32]. Unlike earlier reports that the amount and the M_w of pullulan decreased late in the stationary phase because of the presence of α-amylase secreted into the medium in the batch culture [13, 20, 38], the decline of M_w and M_n of pullulan was not found in the present continuous culture of *A. pullulans* HP-2001. Furthermore the reduction of productivity and decrease in the M_w of pullulan, which are the most undesirable features in production of pullulan by a batch culture, were not found at the steady state in the continuous culture of *A. pullulans* HP-2001. This means that the production of pullulan by continuous culture eliminated most of the undesirable features associated with a decline of productivity and molecular weight. These results suggest that the decrease in the M_w of pullulan in a batch culture may be related to a shortage of nutrients, resulting in secretion of a type of α-amylase (pullulanase): It is highly likely that feeding nutrients into a bioreactor extends the steady state and retards excretion of pullulanase.

Effect of Sucrose Concentration in the Feed Solution on Production of Pullulan

The effect of sucrose concentration in the feed solution on the production of pullulan by *A. pullulans* HP-2001, which were grown in the continuous culture with a dilution rate of 0.015/h, was investigated (Fig. 5). As shown in the figure, average concentrations of pullulan with 100, 150, and 200 g/l sucrose for the steady-state were 69.3 g/l, 86.3 g/l, and 113.5 g/l, respectively. Furthermore, the steady state in the continuous culture fed with 200 g/l sucrose feed solution was maintained longer than that with the feed solutions containing 100 and 150 g/l sucrose. The concentrations of residual sugar in the medium with 100, 150, and 200 g/l sucrose at the steady state were 11.3 g/l, 19.4 g/l, and 37.8 g/l, respectively.

Each continuous culture of *A. pullulans* HP-2001 with different concentrations of sucrose in the feed solution showed a certain period of time of the steady state and its productivity was decreased after the steady state. Continuous systems always have a significant productivity advantage for primary product, but most commercial bioprocesses are batch systems because of genetic instability [33]. The

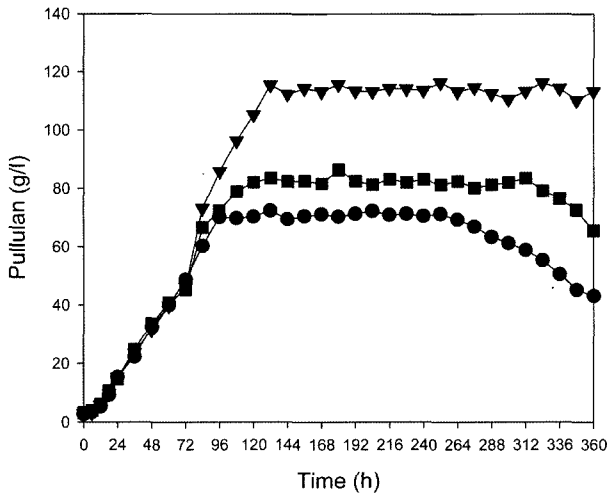

Fig. 5. Effect of sucrose concentration in the feed solution on the production of pullulan by continuous culture of *A. pullulans* HP-2001 (●, 100 g/l; ■, 150 g/l; ▲, 200 g/l sucrose).

decrease of pullulan production during continuous culture may also be due to genetic instability. The mutant strains with higher productivity often grow less than the parental strain. Apparently, the continuous culture imposes strong selection pressure for the most rapidly growing cell [3, 15]. In the continuous culture, a less productive variant will become dominant and decrease productivity.

In conclusion, the present results demonstrated that supplementation of the feed solution with yeast extract as a nitrogen source improved the production of pullulan by *A. pullulans* HP-2001, and the higher concentration of sucrose in the feed solution was attributed to the elaboration of pullulan production and extension of the steady-state. It seems that 200 g/l sucrose in the feed solution would be an economic level to be maintained during continuous culture for a higher production of pullulan with higher molecular weight.

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