Inhibition Effect of Sugar Concentrations on the Cell Growth and the Pullulan production of *Aureobasidium pullulans*

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*Aureobasidium pullulans*의 성장 및 플루란 생산에 미치는 고농도당의 저해효과

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ABSTRACT: For the production of pullulan from the high concentration of sugar, the utilization of sugars by a pullulan-producing fungus, *Aureobasidium pullulans* was examined. *A. pullulans* showed the different utilization patterns for sugars such as sucrose, maltose, and maltotriose. Especially for maltotriose, the hydrolysis of sugar was accompanied by a transferase activity. Glucose and maltose showed the inhibitory effect on the cell growth and the pullulan production at the sugar concentration higher than 0.28 M, but sucrose showed the inhibitory effect at the sugar concentration higher than 0.14 M. Among the sugars examined, sucrose gave the best result for the pullulan production. 27.5 g/l of pullulan was obtained from 5 % sucrose.

KEY WORDS □ *Aureobasidium pullulans*, pullulan production, inhibition effect.

Pullulan is a water soluble neutral glucan which is a linear polymer of maltotriose units connected by α-(1→6) linkages (Bender and Wallenfels, 1961; Bouveng *et al,* 1963). With the increase of pullulan applications to food, drug, and other industries, researches on the production of pullulan with *A. pullulans* have been emphasized (Yuen, 1974).

For the cell growth, *A. pullulans* assimilates D-xylose. L-rhamnose, D-galactose, D-glucose, sucrose, maltose, cellobiose, lactose, inulin, soluble starch, and the non-glycosidic substrates, glycerol and acetate (Ueda *et al.,* 1963; Catley, 1971 a; Cernakova *et al.,* 1980). Among these, glucose, sucrose, fructose, maltose, and xylose were examined for the production of pullulan (Zajic and LeDuy, 1977). However, the detailed reports on the utilization of sugars are few in number. Especially, in polysaccharide fermentations, the utilization of the high concentration of sugars without the decrease of the production rate of polysaccharide has some advantages for the process economy and for saving the solvent used for the recovery of polysaccharide.

As a preliminary experiment for these purposes, this paper shows the utilization patterns of sugars and the inhibitory effects of high sugar concentrations on the cell growth and the pullulan production.

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MATERIALS AND METHODS

Cultivation of microorganism

Ueda's medium (1963) was slightly modified as follows: glucose, 10%; K_2HPO_4, 0.5%; NaCl, 0.1%: $MgSO_4$ $7H_2O$, 0.02%; $(NH_4)_2SO_4$, 0.06%; yeast extract, 0.3% (w/v). Initial pH was adjusted to 7.5 with a concentrated HCl solution. *A. pullulans* IFO 4464 was maintained at 4°C on agar plates containing the medium described. They were transferred to new plates for every week. One loop of *A. pullulans* was transferred to 500 m*l* Erlenmeyer flask containing 100 m*l* of medium and cultivated for 3 days at 27°C, 200 rev/min in shaking incubator (Lab-Line Instruments). 5% of 3 day-culture was transferred and cultivated for 24 hrs in the same conditions described, which was used as an inoculum for main culture by the volume ratio of 5%. The experiments were conducted for Erlenmeyer flask and jar fermentor (New Brunswick Scientific, BioFlo M-30).

Mesurement of dry cell weight and pullulan

4 m*l* of culture broth was diluted to 2 times with distilled water and centrifuged at 10,000 × g for 30 min. Cells harvested were washed with an equal volume of distilled water and dried in an oven at 105°C and weighed. To measure the pullulan, supernatant was collected from the culture broth and followed by the addition of two volumes of ethanol to precipitate exopolysaccharide. The precipitated exopolysaccharide was collected and washed with ethanol and acetone, and dried to a constant weight in an oven at 105°C.

Measurement of sugars

After removal of exopolysaccharide from the supernatant of culture broth by ethanol precipitation, ethanol was evaporated by a rotary evaporator at 50°C and the volume was adjusted to 3 m*l* with distilled water and used for the measurement of residual sugars. Glucose was measured by a glucose diagnostic kit(Sigma Chem. Co.). Reducing sugar was measured by the dinitrosalicylic acid method (Miller, 1959). The total residual sugars were measured by the phenol-sulfuric acid method (Dubois, *et al.* 1956) and fructose, sucrose, maltose were measured by the method of Shin *et al* (1987 a). The utilization of maltotriose was analyzed with an HPLC system (Waters Associates) equipped with Lichrosorb NH_2 (10 μm) column (Merck). The solvent was acetonitrile: water (73:27) and the flow rate was 1.8 m*l*/min.

Measurement of enzyme activities

For the measurement of sucrase activity, the cells cultivated for 24 hrs on sucrose medium were harvested by centrifugation at 12000 × g for 15 min at 4°C and washed once with 50 mM phosphate buffer, pH 6.5. The cells were then resuspended in the same buffer and broken in ice-water bath by sonification for 20 min (Sonifier, Branson Sonic Power Co.). After the breakage, the suspension was centrifuged at 105000 × g for 45 min at 4°C. The precipitated membrane cell wall fraction was washed once more and resuspended in the same buffer. For the measurement of maltase activity, the cells were cultivated on maltose medium and followed the same procedures as described. The supernatant of culture broth, the 105000 × g supernatant, and the particulate fraction were used for measuring the enzyme activities. Reaction mixture containing 0.5 m*l* of 50 mM sugar in 50 mM phosphate buffer, pH 6.5 and 0.5 m*l* of enzyme solution was incubated for 30 min at 37°C and the reaction was stopped by heating at 100°C for 4 min. The glucose produced was measured by glucose-diagnostic kit. One unit of sucrase or maltase activity was defined as the amount of enzyme that releases one micromole of glucose per min under the described conditions. For the detection of transferase activity, the cells were cultivated on maltotriose medium for 72 hrs. The supernatant of culture broth was collected by centrifugation at 12000 × g for 15 min and reacted with 50 mM of sugars in 50 mM phosphate buffer, pH 6.5 at 37°C for 24 hrs. The reaction mixture was analyzed with HPLC system as described above.

Measurement of dissolved oxygen and pH

Content of the dissolved oxygen in culture broth during the cultivation was measured by using the galvanic dissolved oxygen electrode (Vir-Tis Co., Model 6701-0250). The pH of culture broth was measured by using Beckman pH meter

Model 100900.

RESULTS AND DISCUSSION

Kinetics of batch cultivation

The kinetics of batch cultivation of *A. pullulans* on 10% glucose medium is shown in Fig. 1. Maximum dry cell weight and exopolysaccharide obtained were 12 g/l and 20 g/l at 7 day-cultivation. The exopolysaccharide production was not concomitant with cell growth. After 12 hr of cultivation, the cell growth reached the late logarithmic phase but the production of exopolysaccharide just prompted to increase. At this time, total nitrogen content and pH were sharply decreased and the dissolved oxygen was almost exhausted. According to the results of Catley (1971 b), the decreases of nitrogen content and pH were considered as to contribute the formation of exopolysaccharide. But the exhaust of the dissolved oxygen caused the decrease of the production rate of exopolysaccharide (Shin *et al*, 1987 b). The exopolysaccharide fermentation with *A. pullulans* could be divided into two phases according to the pattern of exopolysaccharide production; cell-growing phase and exopolysaccharide-accumulating phase. The differences between the two

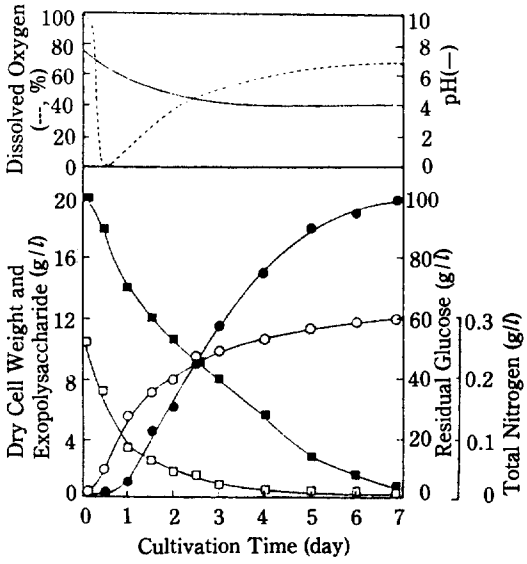

Fig. 1. *Kinetics of batch cultivation of A. pullulans.* Dry cell weight (○), exopolysaccharide (●), residual glucose (■), and nitrogen content (□).

Table 1. *Differences of physiological parameters between the cell-growing and the exopolysaccharide-accumulating phases in batch cultivation.*

Parameter	Cell-growing phase	Exopolysaccharide accumulating phase
pH	5.5-7.5	3.5-5.0
Specific growth rate (hr^{-1})	0.11-0.13	~ 0.0
Specific production rate (g product/g cell hr)	0.02	0.08
Specific oxygen uptake rate (mM O_2/g cell hr)	2.55	0.9
Morphology	Yeast form	Yeast-like and mycelial form

phases are shown in Table 1. Compared with the cell-growing phase, the exopolysaccharide-accumulating phase showed lower pH range 4.0-5.0, nearly zero specific growth rate, 4 times higher specific production rate, and 0.35 times lower specific oxygen uptake rate. Especially during the exopolysaccharide accumulating-phase, yeast-like form of cells changed into the branched mycelial-form in their morphology. Initial pH 7.5 decreased to 4.0 after 3 day-cultivation and thereafter it was maintained constant. The black pigmentation of culture broth was suddenly occurred after 2 day-cultivation. With the proceeding of pigmentation, yeast-like cells called by blastospores were transformed into large swollen cells called by chlamydospores showing the blackness in their cell walls. Black pigment was also observed in the cell-free culture broth. During the cultivation, *A. pullulans* showed distinctive characteristics containing pH change, morphological transformation, black pigmentation, and the pattern of pullulan formation. These phenomena were seemed to be correlated with each other, which is under progress in our laboratory.

Utilization of Sugars

When the utilization patterns of sugars were compared, they showed remarkable differences as shown in Fig. 2 and 3. On the utilization of mal-

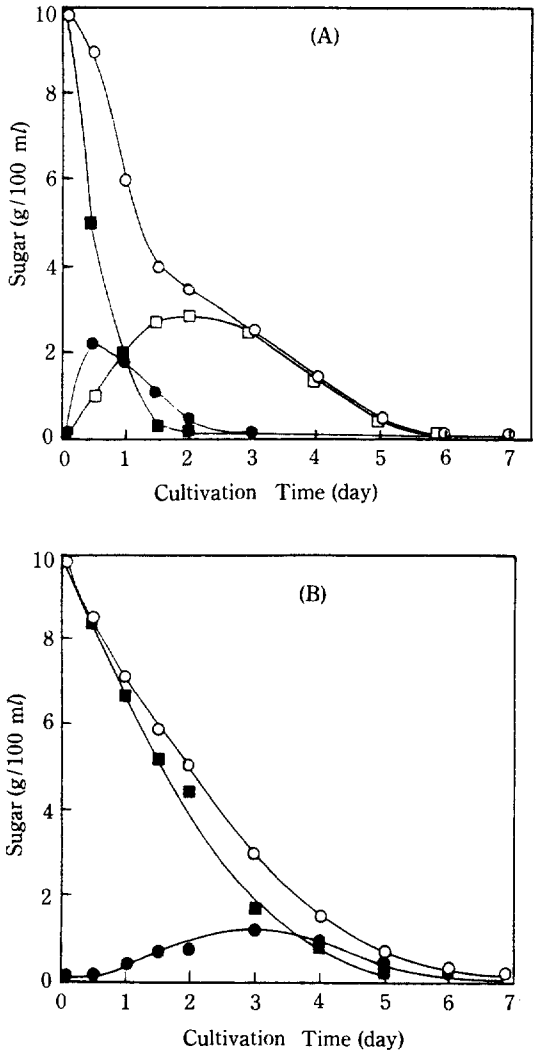

Fig. 2. *Utilizations of sucrose(A) and maltose(B) by A. pullulans. (A) Total residual sugar (○), sucrose (■), glucose (●), and fructose (□). (B) Total residual sugar (○), maltose (■), and glucose (●).*

tose, most of sugars retained in culture broth was maltose at the early phase of cultivation and small amount of glucose was appeared in culture broth at the late phase of cultivation. This result implies that the uptake rate of glucose by cells is at least equal to the hydrolysis rate of maltose or most of the maltose may be taken up by a direct transport system. As shown in Talbe 2, most of the maltase activity was found in cells and small amount was found in the supernatant of culture broth. Extracellular maltase in 1 m*l* culture broth produced

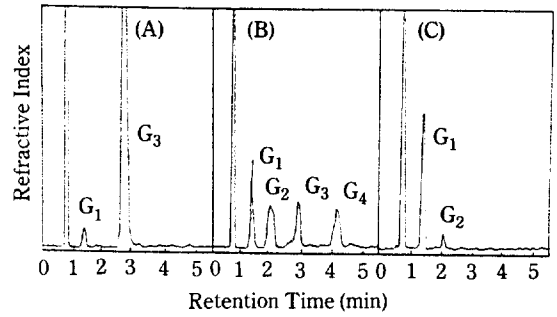

Fig. 3. *Utilization of maltotriose by A. pullulans.* HPLC chromatograms of the culture broths taken at 0 hr (A), 20 hr (B), 60 hr (C). G_1, G_2, G_3, and G_4 represent glucose, maltose, maltotriose, and maltotetraose, respectively.

0.432 mg of glucose from maltose per hour. When *A. pullulans* was cultivated on glucose medium, 1.0 mg of glucose was taken up per 1 m*l* of culture broth per hour. From these results, the glucose uptake rate may be higher than the hydrolysis rate of maltose. However, it is not known whether maltose is taken up by a membrane transport system. The utilization of sucrose was comparable with that of maltose. Most of the sucrose was rapidly converted to the glucose and fructose at the early stage of cultivation and after that, in these monosaccharides, glucose was firstly utilized and then fructose was utilized. As shown in Table 2, sucrase activity at the 24 hr-cultivation was 1.04 units per m*l* of culture broth and evenly distributed in extracellular, intracellular, and particulate fractions. The utilization of maltotriose was investigated with HPLC. After 20 hr-cultivation, glu-

Table 2. *Enzyme activities of A. pullulans*

Enzyme	Fraction	Enzyme Activity (U/m*l* of culture broth)
Sucrase[a]	Extracellular	0.37
	Intracellular	0.34
	Particulate	0.33
Maltase[b]	Extracellular	0.04
	Intracellular	0.04
	Particulate	0.10

a. *A. pullulans* was cultivated for 24 hr on 5% sucrose medium and the culture broth was analyzed.
b. *A. pullulans* was cultivated for 24 hr on 5% maltose medium and the culture broth was analyzed.

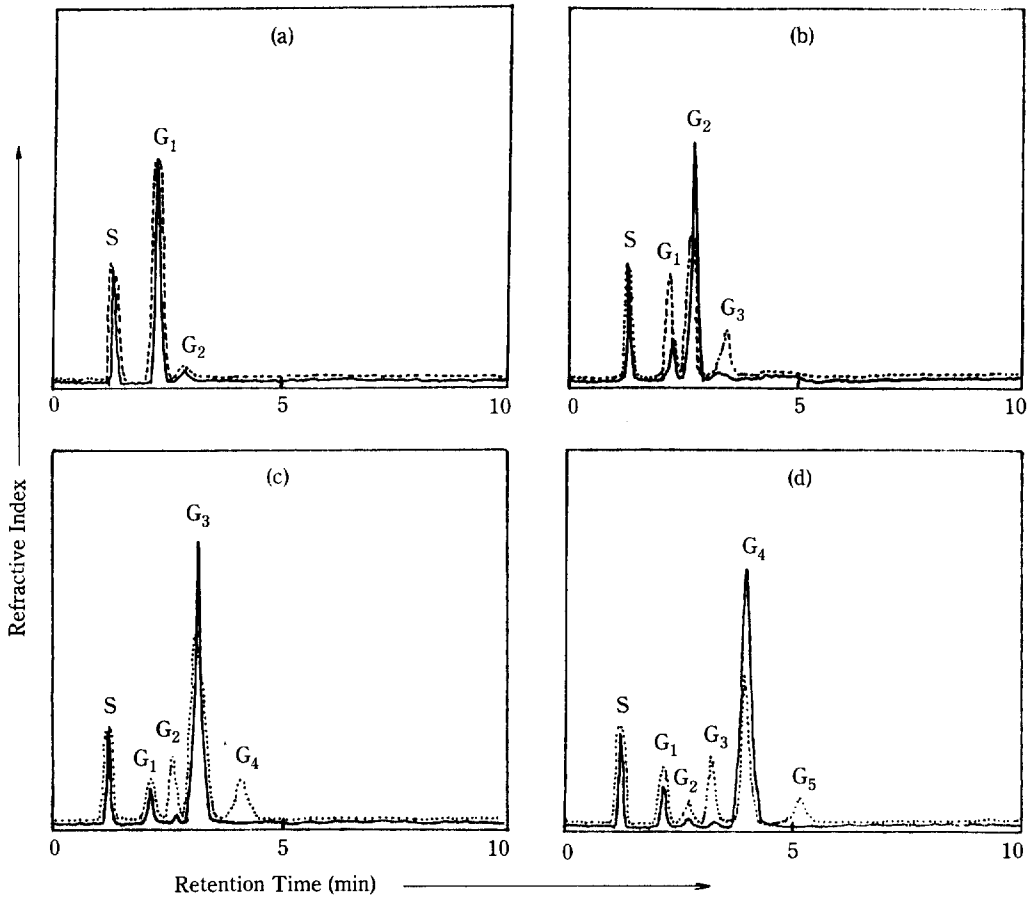

Fig. 4. *Hydrolase and transferase activities of A. pullulans.*

The supernatant of culture broth was reacted with glucose (G_1, a) maltose (G_2, b), maltotriose (G_3, c), and maltotetraose (G_4, d). Reaction mixture was analyzed at 0 hr (—) and 24 hrs (---) incubations. S and G_5 represent solvent and maltopentaose.

cose, maltose, maltotriose, and maltotetraose were observed in culture broth (Fig. 3-B) and after 60 hr-cultivation, most of the sugar remained was glucose and small amount of maltose was detected (Fig. 3-C). Maltotetraose at the early stage of cultivation seemed to be appeared by transferring glucose unit to sugars. To elucidate the transferase activity, *A. pullulans* was cultivated on maltotriose medium and the supernatant of culture broth was reacted with the various sugars. As shown in Fig. 4, when the supernatant was reacted with maltose, maltotriose, and maltotetraose, the supernatant hydrolyzed these sugars in accompanying with the formation of a glucose unit-higher oligosaccharides. But when the supernatant was reacted with glucose, the supernatant did not show the transferase activity at all. From these results, it can be suggested that *A. pullulans* hydrolyzed the maltotriose in accompanying with the transferase activity. Maltotriose, a polymeric unit of pullulan, did not show the inductive effect on pullulan synthesis. When the cell growth and exopolysaccharide production were examined for the various sugars, sucrose gave the best result for the production of exopolysaccharide as shown in Table 3; 27.5 g/*l* of exopolysaccharide was obtained from 5% sucrose.

To understand the effects of sugar concentrations on the cell growth and the exopolysaccharide production, kinetic analysis was carried out. The logistic equation for biomass and the Luedeking-Pirt equation for product formation were introduc-

Table 3. *The cell growth and the exopolysaccharide production of A. pullulans for the various sugars. 5% of sugar was used as a carbon source and 0.1% of yeast extract was used as a nitrogen source.*

Sugar	Dry cell weight (g/l)	Exopolysaccharide (g/l)	Final pH
Glucose	9.5	22.5	4.0
Fructose	8.2	22.0	4.0
Maltose	9.0	19.0	4.2
Sucrose	9.0	27.5	4.0
Maltotriose	9.0	18.0	4.4

ed. These equations were developed by Weiss and Ollis (1980) for the analysis of the microbial polysaccharide fermentations. They are $dX/dt = \mu X(1-X/Xmax)$ and $dP/dt = m(dX/dt) + nX$, where X is biomass concentration at time t (g/l) ; Xmax is maximum attainable biomass concentration in batch fermentation (g/l) ; μ is initial specific growth rate (hr^{-1}); P is product concentration at time t (g/l) ; m is the empirical constant for growth-associated product formation (g product/g cells); and n is the empirical constant for non-growth associated product formation (g product/g cell hr). By following the procedures of the Weiss & Ollis, we calculated the specific growth rate (μ), specific production rate (q_p), m, n, from the empirical data of X, P, Xmax, Xo, and Po. Our empirical data were well fit for these kinetic equations, showing the regression coefficient greater than 0.930. The changes of maximum μ and q_p according to the concentrations of sugar are shown in Fig. 5. For glucose and maltose, the sugar concentrations higher than about 0.28 M and for sucrose, the sugar concentrations higher than about 0.14 M inhibited the cell growth and especially the exopolysaccharide production. The differences in tolerable sugar concentration were apparently considered as to be related with the utilization pattern of sugar. For example, sucrose was

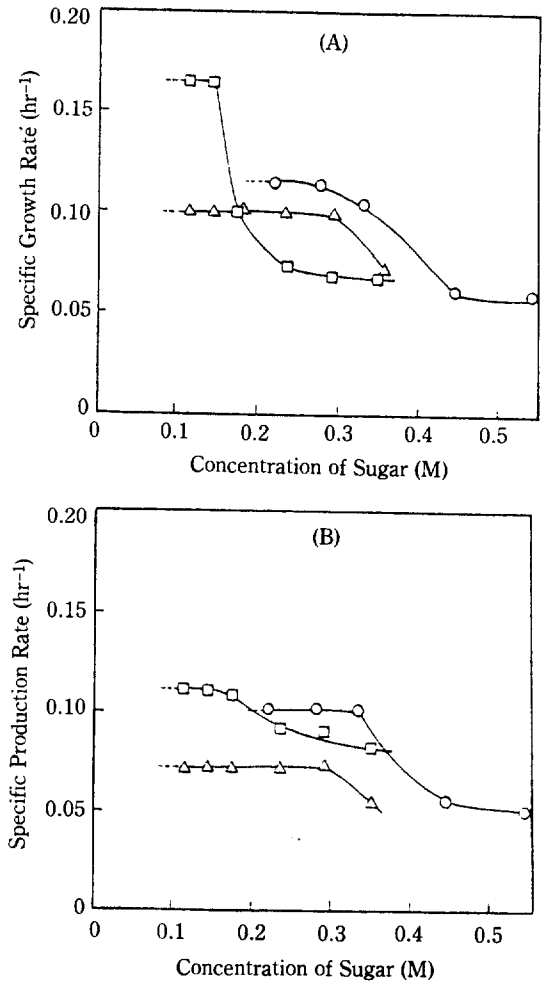

Fig. 5. *Inhibitory effects of high sugar concentrations on the maximum specific growth rate (A) and specific production rate of exopolysaccharide (B). Glucose (○), maltose (△), and sucrose (□).*

rapidly hydrolyzed to glucose and fructose after inoculation, as shown in Figure 2, which means the increase of molar concentration of sugar by two times. For the utilization of high sugar concentration in a batch without the decreases of specific growth rate and specific production rate, the fed-batch fermentation or the isolation of osmotolerant mutants is indicated.

적 요

풀루란을 생산하는 불완전 곰팡이의 일종인 *Aureobasidium pullulans*를 사용하여 고농도 당으로 부터 풀루란을 생산하기 위한 기초연구로서 여러가지 당의 이용 패턴과 당 농도에 따른 균체성장 및 풀루란 생산의 억제 효과를 연구하였다. *A.*

*pullulans*는 sucrose, maltose, maltotriose 배지에서 각기 다른 이용 패턴을 보여주었으며 특히 maltotriose 배지에서는 당의 가수분해와 함께 당의 전이가 일어났다. 당의 농도에 따른 균체성장과 풀루란 생산을 동력학적으로 분석한 결과 glucose와 maltose의 경우 0.28M 이상의 농도에서, sucrose의 경우는 0.14M 이상의 농도에서 비성장속도와 비생산속도가 억제를 받아 감소하였다. 조사한 여러가지 당 중에서 sucrose가 최고의 풀루란 생산성을 보여 5% sucrose로부터 27.5(g/*l*)의 풀루란을 생산할 수 있었다.

사 사

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