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Effect of Dissolved Oxygen Concentration and pH on the Mass Production of High Molecular Weight Pullulan by *Aureobasidium pullulans*

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Abstract The effects of DO and pH on the mass production of pullulan with high molecular weight and the morphology of *A. pullulans* ATCC 42023 were evaluated. *A. pullulans* showed a maximum production of pullulan (11.98 g/l) when the initial pH of the culture broth was 6.5 in a shake-flask culture. In a batch culture, the mixture of a yeast-like and mycelial cell forms was found at a pH of 4.5, and the maximum production of pullulan (13.31 g/l) was obtained. However, a high proportion of high molecular weight pullulan (M.W. >2,000,000) was produced at a pH of 6.5, with a yeast-like morphology. The maximum pullulan production yield (51%) was obtained at a pH noncontrol (initial pH 6.5) and DO control (above 50%) condition. Pullulan degrading enzyme was activated when the pH of the broth was lower than 5.0 and the portion of low molecular weight pullulan was increased. The formation of a black pigment was observed at an initial stationary phase, at 40 h of fermentation. Therefore, the fermentation should be carried out in a pH noncontrol (initial pH of 6.5) and DO control (above 50%) condition, and should be harvested before reaching the stationary phase (around 40 h) for the production of high molecular weight pullulan.

Key words: *Aureobasidium pullulans*, pullulan, dissolved oxygen concentration, pH, molecular weight

Many polysaccharides produced by microorganisms have been studied, including xanthan [18], curdlan [9], pullulan [4], gellan [14], welan [5], and rhamsan [13]. Pullulan is an extracellular water-soluble microbial polysaccharide produced by the yeast-like fungus *Aureobasidium pullulans*. It mainly consists of maltotriose units interconnected via $\alpha(1\rightarrow6)$ linkages [4].

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A number of potential applications have been reported for pullulan due to its transparent, oil resistant, and oxygen impermeable film-forming properties. Pullulan could be used as a coating for packing materials, in calorie food formulations, cosmetic emulsions, and other industrial and medicinal applications [4]. Important parameters for pullulan synthesis are temperature [11], pH of the medium [8], oxygen supply [20], nitrogen concentration [16], and carbon source [21, 22]. Among these parameters, the pH of the culture broth is one of the main factors influencing not only the production of pullulan, but also the morphology of *A. pullulans* [17]. Besides the yield of pullulan production, the important parameter for the commercial application of pullulan is the molecular weight. Pullulan with a high molecular weight has a high viscosity, thus, it is more valuable than that with a low molecular weight.

The factors that control the rate of pullulan synthesis and produce high molecular weight pullulan have not been well studied. Several factors for the synthesis and secretion mechanism of pullulan and cellular metabolism of *A. pullulans* have been reported [8]. The pH of the culture broth influences not only the production of pullulan, but also the morphology of *A. pullulans*. The mycelial form and yeast-like form were predominant at pH 2.0–2.5 and pH 6.0–8.0, respectively. There were several reports on the relationship between the production of pullulan and its morphology [17]. However, these reports showed somewhat conflicting results. Therefore, further study on the relationship between the morphology and pullulan production is necessary to increase the yield of pullulan and to obtain high molecular weight pullulan.

The viscosity of the fermentation broth decreases due to the decrease in the average molecular weight of the pullulan from 3×10^6–6×10^6 to 1×10^5–2×10^5 by the action of pullulanase [15]. Pullulanase is a debranching enzyme

which hydrolyses the α-1,6-glycosidic linkages in pullulan and other amylaceous polysaccharides [7].

In this study, the effects of dissolved oxygen and pH on the morphology, the synthesis of pullulan, and the molecular weight distribution of pullulan during the fermentation process were evaluated to obtain the optimum pullulan fermentation condition.

MATERIALS AND METHODS

Microorganism and Medium Compositions

Aureobasidium pullulans ATCC 42023 [19] was used as a fermentation organism. The medium used for cell growth and pullulan production contained the following composition (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, 2.5; and glucose, 50 [6]. The medium was adjusted to pH 6.5 with 1 N NaOH.

Culture in the Shake-Flasks

Cultures were grown in a 50 ml medium in 250-ml flasks for 2 days at 28°C in a rotary shaking incubator at 200 rpm. These cultures were used to inoculate (5%, v/v) 150 ml of medium held in 500-ml shake-flasks. These cultures were then incubated for 5 days under the same conditions used for the starter cultures. Samples were periodically withdrawn from the cultures to determine cell growth and pullulan production. Sets of shake-flasks were prepared at different initial pH levels in order to evaluate the influence of the initial pH on the kinetic aspects of pullulan production. The shake-flasks containing 150 ml of production medium, at different initial pH levels (2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5), were prepared and incubated at 28°C in a rotary shaking incubator at 200 rpm for 5 days.

Fermentation Conditions

The seed cultures were incubated for 2 days at 28°C with 200 rpm of agitation and used as an inoculum (10%, v/v) for 1.5 l of medium containing glucose (5%, w/v) in a 2.5-l fermenter (Model BioflowIII, New Brunswick Scientific Instrument Co., U.S.A.). The effect of the pH was evaluated by controlling the pH at 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5. The fermentation with the pH control was carried out at 28°C, 500 rpm, and 0.5 vvm for 5 days. Three-hundred ml of cultures were inoculated in a 5-l fermenter (Model KF-5, Korea Fermenter Company Ltd., Inchon, Korea). Fermentation was carried out for 6 days under a pH or O_2 control. The fermenter was operated at 28°C. The impeller speed was maintained constant at 500 rpm, except otherwise stated.

Measurement of Dry Cell Weight and Pullulan Production

The fermentation broth was centrifuged at 400 ×g for 20 min at 4°C to remove the cells. To determine the biomass weight, the cells were washed with distilled water and dried at 105°C until the weight was constant. To measure the amount of pullulan from the broth, the supernatant of culture broth was collected and 2 volumes of 95% ethanol were added. Then, the mixture was stored at 4°C for 24 h to precipitate the crude pullulan. The precipitated material was separated by centrifugation at 400 ×g for 20 min and dried at 105°C until the weight was constant.

Determination of Molecular Weight by Gel Permeation Chromatography (GPC)

The average molecular weights of the pullulan were determined by the GPC system equipped with HP 1100 series 20RBAX PSM HPSEC columns (M.W. range of 1×10^2 to 1×10^7 dal.) and an RI detector.

Pullulan standards with narrow polydispersity and molecular weights ranging from 5.80×10^3 to 1.6×10^6 were used to construct a calibration curve. Water was used as the 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.

Determination of Pullulanase Activity

The activities of standard pullulanase (Sigma Co. St. Louis, U.S.A.) and crude pullulanase obtained from the fermentation broth of the pullulan were determined at various pH levels.

The effect of the pH on the pullulanase activity of the fermentation broth was determined by using the standard procedure [3] and appropriate buffers. For pH 2.0 to 3.0, a 50 mM citrate buffer; for pH 4.0 to 5.0, a 50 mM acetate buffer; and for pH ranging from 6.0 to 8.0, 50 mM phosphate buffers were used.

The crude pullulanase samples from the fermentation broths were collected at two different growth stages to determine the pullulanase activation during the growth phases. The pullulanase from fermentation broths obtained from 1 day (24 h), logarithmic phase, and 3 days (72 h), stationary phase, were named Enzyme-1 and Enzyme-3, respectively. To obtain the pullulanase, the culture broth was centrifuged at 400 ×g for 20 min at 4°C to remove the cell pellet. Aliquots (500 ml) of culture supernatant were treated at 4°C with ammonium sulfate at 80% saturation. The precipitate was obtained by centrifugation at 400 ×g for 20 min at 4°C. The pellet was resuspended in distilled water and dialyzed against 2 l of distilled water for 24 h in a dialysis tubing (molecular weight cut-off, 3,500; Spectrum Medical Industries Inc., Houston, U.S.A.). The protein concentration was determined by the method of Lowry *et al.* [10] using bovine serum albumin as the standard.

To determine the optimum pH for the pullulanase activity, 900 μl of pullulan (1 mg/ml) from the fermentation, and 100 μl of standard pure pullulanase (0.1 mg/ml) or isolated pullulanase (0.1 mg/ml) were incubated at 25°C

for 48 h. The released reducing sugars were determined by the dinitrosalicylic acid method [12]. One unit of activity was defined as the amount of enzyme that catalyzed to release 1 μmol of maltotriose (measured as glucose) per min.

RESULTS AND DISCUSSION

Optimum Initial pH of the Pullulan Production

The pH of the medium was changed to obtain optimum conditions under which pullulan could be produced. The maximum cell growth of *A. pullulans* with an initial pH of 3.5 was achieved at the level of 6.87 g/l with a yield coefficient of 0.14 (Fig. 1). However, the pullulan production increased from this initial pH up to 6.5. The maximum pullulan production, 11.98 g/l, was obtained in the culture grown at an initial pH of 6.5 with a yield coefficient of 0.24 (Fig. 1). This result indicated that the optimum initial pH for pullulan production was different from that of the cell growth. Also, the optimum pH of the cell growth and pullulan production were different from the initial pH, because the pH of the culture broth changed during the cultivation. The culture with the initial pH of 6.5 showed a medium pH of 4.5 after 24 h of culture (data not shown). This result agreed with those of Badr-Eldin *et al.* [1] who also reported that pullulan production was the highest with an initial pH of 6.5.

Effects of pH on the Yield of Pullulan and the Morphology of *A. pullulans*

A. pullulans was cultured in a 2.5-l fermenter containing 1.5 l of medium, with the pH controlled at 2.5 to 7.5 by the addition of 1 N HCl or 1 N NaOH. The maximum cell growth of *A. pullulans* at a pH of 6.5 was achieved at the level of 16.55 g/l, with a yield coefficient of 0.33 (Fig. 2). However, the maximum pullulan production was obtained at a pH of 4.5, and the pullulan concentration was 13.31 g/l with a yield coefficient of 0.27 and a specific yield of 1.5 g/g. These results agreed with those of Shin *et al.* [17].

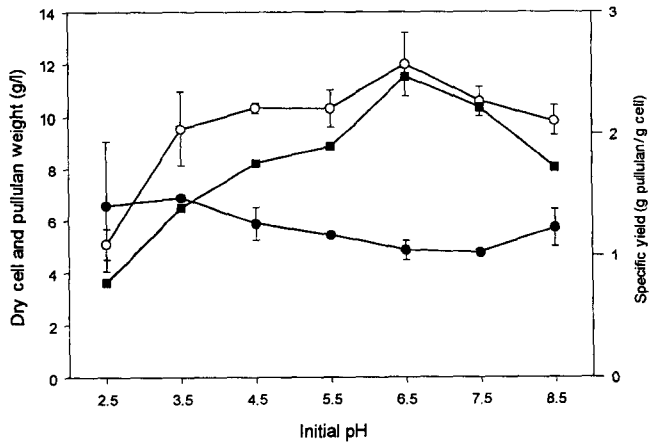

Fig. 1. Effect of initial pH on the production of pullulan and specific yields on pullulan production by *A. pullulans* in shake-flasks.

The data were obtained from 5-day culture grown at 28°C and the values were based on results of triplicate experiments. The different initial pH was adjusted to 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 before sterilization. (-●-): Dry Cell Weight (g/l). (-○-): Pullulan Weight (g/l). (-■-): Specific Yield (g pullulan/ g cell).

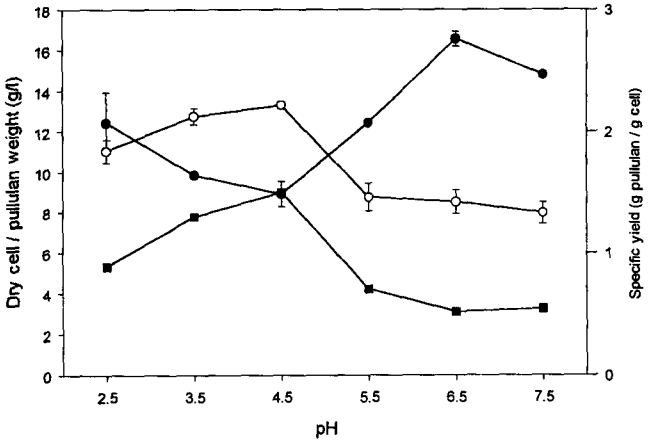

Fig. 2. Effect of pH on the production of pullulan and specific yields on pullulan production by *A. pullulans* in 2.5-l fermenter.

The impeller speed, air flow rate, and temperature for culture were 500 rpm, 0.5 vvm, and 28°C, respectively. The constant pH was adjusted to 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5 before sterilization. The values were based on results of triplicate experiments. (-●-): Dry Cell Weight (g/l). (-○-): Pullulan Weight (g/l). (-■-): Specific Yield (g pullulan/ g cell).

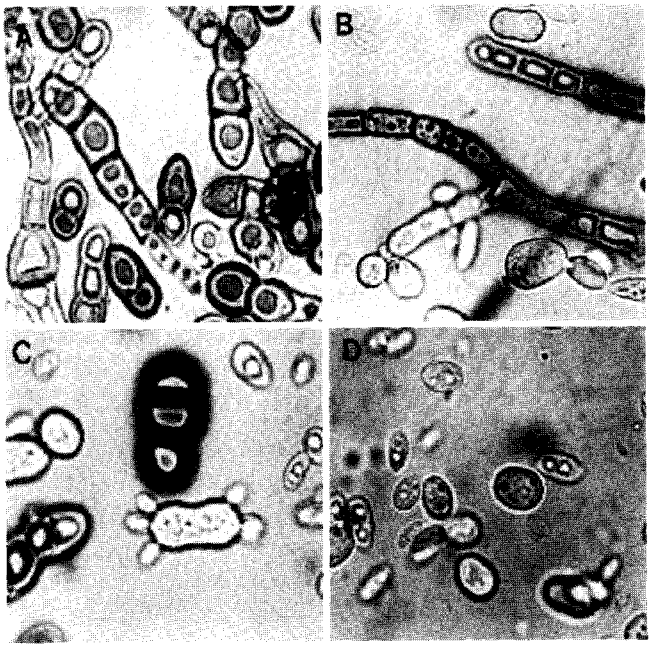

Fig. 3. The different morphologies of the *A. pullulans* at various pHs.

(A) pH 2.5; (B) pH 4.5; (C) pH 6.5; (D) pH 7.5.

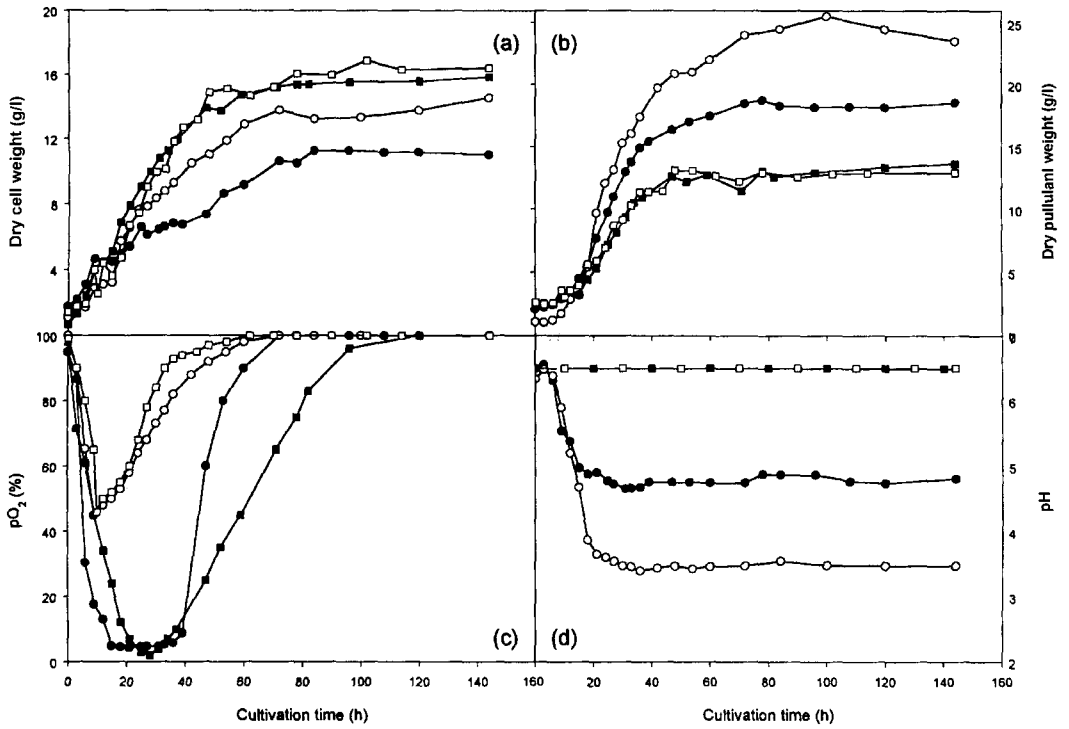

Fig. 4. Effect of pH control and dissolved oxygen concentration control on the production of pullulan and pH by *A. pullulans* in 5-l fermenter. Cultivated at different culture conditions.
(a) Dry cell weight; (b) dry pullulan weight; (c) pO2; (d) pH. (□): Constant pH 6.5 and DO control to 50% of pO_2 (pH control and DO control). (■): Constant pH 6.5 and without DO control (pH control and DO noncontrol). (○): Initial pH 6.5 and with DO control to 50% of pO_2 (pH noncontrol and DO control). (●): Initial pH 6.5 and without DO control (pH noncontrol and DO noncontrol).

The morphology of the cell at various pH levels was evaluated by a microscope. Culture pH was the most important parameter to significantly affect morphological change [2]. Cells at pH 2.5 showed mycelial growth (Fig. 3A), however, the mycelial form started to change to a yeast-like single cell at pH 4.5 (Fig. 3B). Further increase of pH increased the portion of the yeast-like cell as shown in Figs. 3C and 3D. The cell showed a high growth rate during single cell morphology at pH 6 to 7. However, the pH decrease of the culture broth induced the morphological change to mycelia. Due to the linear mycelial growth, the cell mass yield coefficient decreased. When the mycelial growth started, by the pH decrease to 4.5, the environmental condition for the cell became unfavorable, thus leading to exopolysaccharide pullulan production.

Effect of pH and DO on Cell Growth and Production of Pullulan

Cell growth and pullulan production from *A. pullulans* were determined at various culture conditions with dissolved oxygen (DO) and pH as variables. DO and pH values were monitored (Fig. 4), and the final yields and

Table 1. Effect of dissolved oxygen concentration and pH control on the cell mass and pullulan productivity.

pH	DO	Final pH	Cell			Pullulan		
			Concentration (g/l)	Yield ($Y_{X/S}$, g/g)	Specific growth rate μ_{max} (1/h)	Concentration (g/l)	Yield (Y_{PS}, g/g)	Productivity (g/l/d)
6.5[a]	50[c]	6.50	16.86	0.34	0.103	13.10	0.26	2.64
6.5[a]	–[d]	6.50	15.84	0.32	0.101	13.62	0.27	2.64
–[b]	50[c]	3.47	14.26	0.29	0.099	25.54	0.51	5.04
–[b]	–[d]	4.81	11.27	0.23	0.134	18.73	0.37	3.84

[a]pH control condition: pH was controlled to 6.5.
[b]pH noncontrol condition: initial pH was adjusted to 6.5.
[c]DO control condition: DO value was maintained above 50% by increasing agitation speed.
[d]DO noncontrol condition: aeration was set to 0.5 vvm and 500 rpm of agitation speed.

productivities of the cell and pullulan were summarized in Table 1.

The DO (Fig. 4c; blank) was controlled with agitation speed (500 rpm to 800 rpm) to maintain O_2 concentration above 50%, and the pH (Fig. 4d; square) was controlled to 6.5 with 1 N HCl and 1 N NaOH. Concentrations of biomass and pullulan increased steadily during 50 h of fermentation as shown in Figs. 4a and 4b, respectively.

At a constant pH condition of 6.5, the cell growth and pullulan productions in each case showed similar patterns regardless of dissolved oxygen concentrations (Figs. 4a and 4b; square). The cultures with pH control conditions showed higher cell growth than those without pH control, however, the cultures showed lower pullulan productions than those without pH control. When the pH of the culture was maintained at 6.5 throughout the fermentation period, the oxygen concentration did not influence the cell growth and pullulan production. A constant pH level of 6.5 might provide a proper condition for the cell to proliferate. Most of the carbon source allocated to cell mass production caused less production of the pullulan.

In the case of DO noncontrol, oxygen was provided at a constant rate (0.5 vvm, 500 rpm) and oxygen concentration rapidly decreased to less than 10% in 20 h. In the case of the DO control condition, dissolved oxygen concentration decreased to less than 50% in 10 h, thus the agitation speed was increased to 800 rpm to increase the oxygen concentration to higher than 50%.

At pH noncontrol (initial pH of 6.5) conditions with and without DO control, the maximum cell densities were low compared to those of the pH control conditions. However, the production of pullulan increased compared to those of the pH control conditions. (Figs. 1a and 1b, circle). The maximum pullulan production was achieved at an amount of 25.54 g/l, and the yield coefficient ($Y_{P/S}$) increased to 0.51 with the DO control (Fig. 4b, Table 1).

The final yields and productivity are shown in Table 1. Oxygen is necessary for the biosynthesis of pullulan by *A. pullulans*. High pullulan yield and synthesis rates are associated with high oxygen concentration. The results were in accordance with the fact that the rate of pullulan synthesis and the yield of pullulan were proportional to oxygen availability. The final pH of the culture with a DO control and without a pH control (Fig. 4d, Table 1.) was 3.47, which was lower than the pH of 4.81 in the DO noncontrol and the pH noncontrol conditions. The condition with a pH of 3.5 was not proper for the growth of *A. pullulans*. However, this accelerated the production of pullulan from glucose. Therefore, the mass production of pullulan was obtained when the pH reached a level of 3.5 in the pH noncontrol, and oxygen concentration was maintained higher than 50%. However, the cell growth and the pullulan production were retarded due to lack of oxygen in the pH noncontrol and the DO noncontrol conditions.

Molecular Weight Distribution of Pullulan and Pullulan Degrading Enzyme Activity

A high molecular weight pullulan above 2.0×10^6 is important for industrial applications. The molecular weight of pullulan could depend on both culture conditions and the strain used [15, 21].

The molecular weight distributions of pullulan produced at various conditions are shown in Fig. 5. The proportion of high molecular weight pullulan increased steadily at the logarithmic phase (40 h) in each condition. The total amounts of pullulan in the pH noncontrol conditions (Figs. 5c and 5d) were much higher than those of the pH control conditions (Figs. 5a and 5b). In the case of the pH noncontrol condition, the portion of high molecular weight pullulan was at a maximum level of 6.02 g/l after 30 h of fermentation (Fig. 5c). However, after reaching the maximum

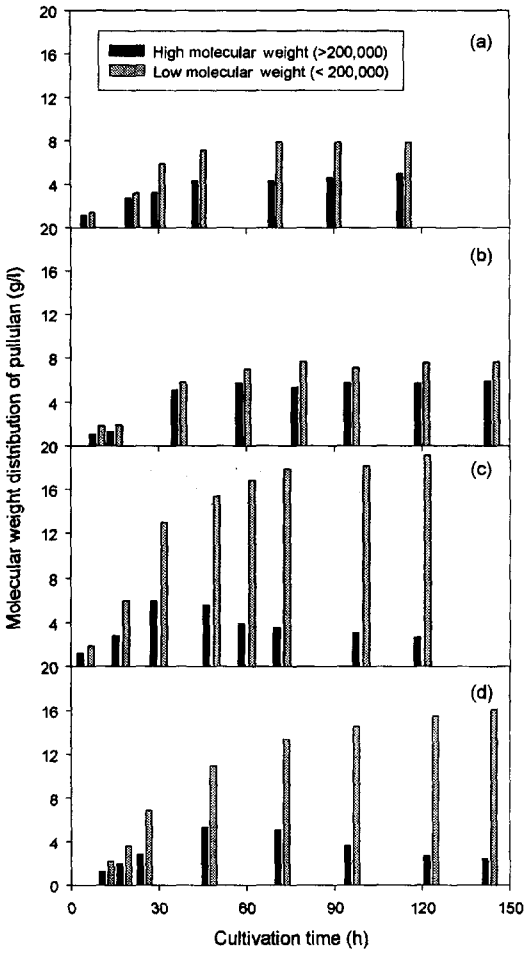

Fig. 5. Pullulan concentration as a function of culture time.
(a) Constant pH 6.5 and DO control to 50% of pO_2 (pH control and DO control), (b) constant pH 6.5 and without DO control (pH control and DO noncontrol), (c) initial pH 6.5 and with DO control to 50% of pO_2 (pH noncontrol and DO control), (d) initial pH 6.5 and DO control (pH noncontrol and DO noncontrol)). (■), High molecular weight (>200,000); (□), low molecular weight (<200,000).

level, the amount of high molecular weight portion of pullulan decreased and, consequently, the low molecular weight portion increased. In the case of a constant pH of 6.5, the portion of high molecular weight pullulan reached a high level of 5.7 g/l at 59 h and maintained the high molecular weight pullulan for 150 h (Fig. 5b).

This indicated that the cells at a high pH produced high molecular weight pullulan, but the yield coefficient of pullulan was low. The reason for the decrease in the high molecular weight portion of pullulan at low pH was likely the pullulanase activation at low pH levels and acid hydrolysis during low pH conditions.

The amount of high molecular weight pullulan produced in a pH control condition (pH 6.5) was maintained at the level throughout the fermentation period. The molecular weight shift in the pH noncontrol condition was caused by the activation of pullulanase. As shown in Fig. 6, standard pullulanase showed optimum activity at pH 4.0–8.0, and the crude pullulanase from a fermentation broth showed optimum activity at a pH of 3.0–4.0. Crude pullulanase from the fermentation broth showed 70% of the pure pullulanase activity.

Generally, pullulanase is one of the starch debranching enzymes, and the optimum pH for activity is in the range of 5.0–7.0. However, crude pullulanase in this study showed optimum activity at pH 3.0 (Fig. 6). Therefore, pH decreases in the culture with a pH noncontrol and a DO control condition promoted the degradation of high molecular weight pullulan. In contrast, the molecular weight of pullulan produced in the pH control condition, pH 6.5, could maintain a high molecular weight portion of pullulan throughout the fermentation process.

During the fermentation with *A. pullulans*, the formation of a black pigment was observed at the initial stationary stage; the pigment is synthesized through the pentaketide pathway in which 1,8 dihydroxynaphthalene is the direct precursor [20]. It has been reported [16] that melanin pigment appears between 2 and 4 days of fermentation. The strain used in the present study produced melanin at the initial stationary phase of 40 h of fermentation, at which the production of the high molecular weight pullulan reached the maximum level.

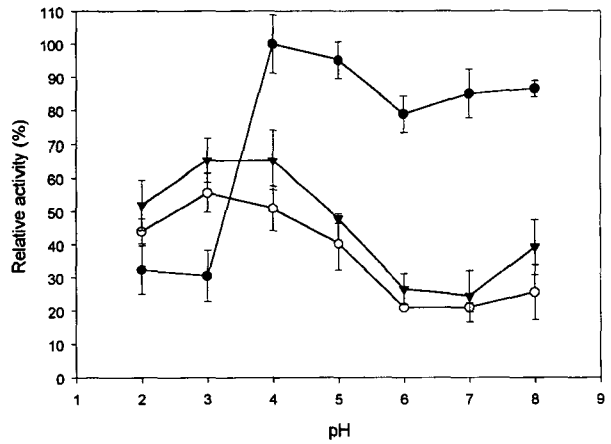

Fig. 6. Effect of pH on the activity of enzyme from culture broth by *A. pullulans* and on pullulanase activity.
Treated pullulan with pure pullulanase (●), crude enzyme solution with fermentation time of 1 day (Enzyme-1) (○), crude enzyme solution with fermentation time of 3 days (Enzyme-3) (▼).

In conclusion, the maximum production yield of pullulan was obtained from the culture of *A. pullulans* with pH noncontrol (initial pH 6.5) and DO control conditions. In addition to the pullulan yield, the fermentation time should be less than 40 h to prevent pigment formation. Therefore, the optimum conditions for the production of high molecular weight pullulan without pigment is suggested to be pH noncontrol (initial pH 6.5) and a DO control (above 50%) condition with 40 h of fermentation time.

REFERENCES

1. Badr-Eldin, S. M., O. M. El-Tayeb, E. G. El-Masry, O. A. Mohamad, and O. A. A. El-Rahman, 1994. Polysaccharide production by *Aureobasidium pullulans*: Factors affecting polysaccharide formation. *World J. Microbiol. Biotechnol.* **10:** 423–426.
2. Bae, J. T., J. Sinha, J. P. Park, C. H. Dong, and J. W. Yun, 2000. Optimization of submerged culture conditions for exo-biopolymer production by *Paecilomyces japonica*. *J. Microbiol. Biotechnol.* **10:** 482–487.
3. Bollag, D. M., M. D. Rozycki, and S. T. Edelstein. 1996. Ammonium sulfate precipitation, pp. 91–93. *In*: *Protein Methods*. 2nd ed. John Wiley and Sons, New York, U.S.A.
4. Deshpande, M. S., V. B. Rale, and T. M. Lynch. 1992. *Aureobasidium pullulans* in applied microbiology: A status report. *Enzyme Microb. Technol.* **14:** 514–527.
5. Hember, M. W. N. and E. R. Morris. 1995. Solubility, solution rheology and salt-induced gelation of welan polysaccharide in organic solvents. *Carbohydr. Polym.* **27:** 23–36.
6. Kim, J. H., M. R. Kim, J. H. Lee, J. W. Lee, and S. K. Kim. 2000. Production of high molecular weight pullulan by *Aureobasidium pullulans* using glucosamine. *Biotechnol. Lett.* **22:** 987–990.
7. Koch, R., F. Cangannella, H. Hippe, K. D. Kahnke, and G. Antranikian. 1997. Purification and properties of a thermostable pullulanase from a newly isolated thermophilic anaerobic bacterium, *Fervidobacterium pennavrans* Ven5. *Appl. Environ. Microbiol.* **63:** 1088–1094.
8. Lacroix, C., A. LeDuy, G. Noel, and L. Choplin. 1985. Effect of pH on the batch fermentation of pullulan from sucrose medium. *Biotechnol. Bioeng.* **27:** 202–207.
9. Lee, J. W., W. G. Yeomans, A. L. Aqllen, D. L. Kaplan, F. Deng, and R. A. Gross. 1997. Exopolymers from curdlan production: Incorporation of glucose-related sugars by *Agrobacterium* sp. Strain ATCC 31749. *Can. J. Microbiol.* **43:** 149–156.

10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 256–275.
11. McNeil, B. and B. Kristiansen. 1990. Temperature effects on polysaccharide formation by *Aureobasidium pullulans* in stirred tanks. *Enzyme Microb. Technol.* **12:** 521–526.
12. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426–428.
13. Morris, E. R., M. G. E. Gothard, M. W. N. Hember, C. E. Manning, and G. Robinson. 1996. Conformational and rheological transitions of welan, rhamsan and acylated gellan. *Carbohydr. Polym.* **30:** 165–175.
14. Paulsson, M., H. Hagerstrom, and K. Edsman. 1999. Rheological studies of the gelation of deacetylated gellan gum in physiological conditions. *Eur. J. Pharm. Sci.* **9:** 99–105.
15. Pollock, T. J., L. Throne, and R. W. Armentrout. 1992. Isolation of new *Aureobasidium* strains that produce high-molecular-weight pullulan with reduced pigmentation. *Appl. Environ. Microbiol.* **58:** 877–883.
16. Seviour, R. J. and B. Kristiansen. 1983. Effect of ammonium ion concentration on polysaccharide production by *Aureobasidium pullulans* in batch culture. *Eur. J. Appl. Microbiol. Biotechnol.* **17:** 178–181.
17. Shin, Y. C. and S. M. Byun. 1991. Effect of pH on the elaboration of pullulan and the morphology of *Aureobasidium pullulans*. *Kor. J. Appl. Microbiol. Biotechnol.* **19:** 193–199.
18. Son, B. S., S. K. Park, S. K. Kang, S. W. Lee, and N. K. Sung. 1995. Rheological properties of exopolysaccharide produced by *Xanthomonas* sp. EPS-1. *Kor. J. Appl. Microbiol. Biotechnol.* **23:** 269–274.
19. Ueda, S., K. Fujita, K. Komatsu, and Z. Nakashima. 1963. Polysaccharide produced by the genus *Pullularia*. *Appl. Microbiol.* **11:** 211–215.
20. Wecker, A. and U. Onken. 1991. Influence of dissolved oxygen concentration and shear rate on the production of pullulan by *Aureobasidium pullulans*. *Biotechnol. Lett.* **13:** 155–160.
21. Wiley, B. J., D. H. Ball, S. M. Arcidiacono, J. M. Mayer, and D. L. Kaplan. 1993. Control of molecular weight distribution of the biopolymer pullulan produced by *Aureobasidium pullulans*. *J. Environ. Polym. Degrad.* **1:** 3–9.
22. Youssef, F., C. G. Briliaderis, and T. Roukas. 1998. Enhancement of pullulan production by *Aureobasidium pullulans* in batch culture using olive oil and sucrose as carbon sources. *Appl. Biochem. Biotechnol.* **74:** 13–80.