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Effects of pH on the Elaboration of Pullulan and the Morphology of *Aureobasidium pullulans*

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배양액의 pH가 *Aureobasidium pullulans*의 풀루란 생성과 형태에 미치는 영향

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Abstract — The effects of pH on the cell growth, the elaboration of pullulan, and the morphology of *Aureobasidium pullulans* IFO 4464 were examined. *A. pullulans* grew in yeast-like form at constant pH 7.5 and in mycelial form at constant pH 2.5. At the both pH conditions, the elaboration of pullulan was very low, about 6.0~6.5 *g/l*. The mixture of yeast-like form and mycelial form of cells was found at the constant pH 4.5, at which condition, the elaboration of pullulan was high, about 24.5 *g/l*. The pH shift experiments showed that the specific production rates of pullulan were 0.048 (hr^{-1}) for the mycelial form and 0.058 (hr^{-1}) for the yeast-like form, which indicated that the yeast-like form has the similar, only slightly higher, biosynthetic activity of pullulan to the mycelial form at pH 4.5 and the pH of culture broth is more important factor for the elaboration of pullulan than the morphology of *A. pullulans*.

Pullulan is a water soluble neutral glucan which is a linear polymer of maltotriose units connected by α-(1-6) linkages (1-4). With the increase of pullulan applications to food, drug, and other industries, researches on the production of pullulan with *A. pullulans* have been emphasized (5).

The factors that control the rate of pullulan synthesis remain obscure, although a number of parameters have been identified that influence production, either by acting on the synthesis or excretion mechanism or modifying cellular metabolism (6-9). Among them, the effects of pH were the most seriously examined (10-12). The pH of culture broth influences not only the elaboration 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. This morphological change was considered as to be related with the pullulan biosynthesis (13, 14). The yeast-like form separated by centrifugation or filtration through nylon mesh (pore size, 45 μm) elaborated the more pullulan than the mycelial form. This result was also confirmed by the yeast-like form mutant. But Lacroix *et al.* (15) reported that biomass obtained at pH 2.0~2.5 could be successfully used for the pullulan production by the pH shift to 4.0~5.0, with nearly the same efficiency as shown by the biomass obtained at pH 5.0~6.0. However, they

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did not discuss the relationship between the morphological change and the pullulan elaboration. Assuming that the biomass obtained at pH 2.5 was predominantly mycelial form as previously reported, their result conflicted with the other results representing that the mycelial form has the lower production yield of pullulan than the yeast-like form. Therefore, further research on the relationship between the morphology and the pullulan elaboration is required.

For the production of pullulan, we investigated the utilization of carbon sources, physiology of *A. pullulans*, rheology of culture broth, and fermentation mode (16-19). In this paper, we examined the effects of culture pH on the morphology and the elaboration of pullulan of *A. pullulans* in detail, and discussed the metabolic activities of mycelial form and yeast-like form of *A. pullulans* for the pullulan elaboration.

Materials and Methods

Microorganism and inoculum

A. pullulans IFO 4464 was purchased from Institute for Fermentation at Osaka, Japan. It was maintained at 4℃ on agar plates containing the medium as follows: glucose, 10%; K_2HPO_4, 0.5%; NaCl, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.02%; $(NH_4)_2SO_4$, 0.06%; yeast extract, 0.3%; and agar, 2% (w/v). The initial pH of culture broth was adjusted to 7.5 with a concentrated HCl solution. *A. pullulans* was serially transferred to fresh plates for every week throughout the experiments. One loop of *A. pullulans* was transferred to 500 m*l* Erlenmeyer flask containing 100 m*l* of the medium and cultivated for 3 days at 27℃, 200 rpm in the shaking incubator (Lab-Line Instruments Co.). 5 m*l* of 3 days-culture was transferred and cultivated for 24 hr at the same conditions, which was used as inoculum for main culture by the volume ratio of 5(%).

Cultivation of microorganism

The experiments were conducted for 500 m*l* Erlenmeyer flask containing 100 m*l* of the specified medium. *A. pullulans* was cultivated at 27℃, 200 rpm for seven days and 5 m*l* of culture broth was sampled at every day of cultivation. The controlled pH experiments were conducted for a jar fermentor (New Brunswick Scientific, MicroFerm) containing 3 *l* of medium under the conditions of 27℃, 400 rpm, and 1 vvm (volume of air input per working volume per min).

Measurement of dry cell weight and exopolysaccharide

4 m*l* of culture broth was diluted with two volume of distilled water and centrifuged at 10,000×g for 30 min. The cells harvested were washed with equal volume of distilled water and the washed cells were dried to a constant weight at 105℃. To measure the exopolysaccharide, the supernant was collected and followed the addition of two volumes of absolute ethanol. The precipitated exopolysaccharide was collected by centrifugation at 5,000×g for 15 min and washed twice with ethanol and acetone. The washed exopolysaccharide was dried to a constant weight in an oven at 105℃.

Thin layer chromatography (TLC)

To identify and quantify the pullulan from the exopolysaccharide produced, 10 mg of exopolysaccharides was dissolved in 1 m*l* of distilled water and hydrolyzed with 0.5 unites of pullulanase (Sigma Chemical Co.) at 25℃ for 12 hr. The hydrolysates were chromatographed on silica gel plates (Eastman-Kodak) in a solvent system containing ethyl acetate, pyridine, and water (10: 4: 3). Spots were visualized by spraying the ethanolic sulfuric acid reagent (20% sulfuric acid in ethanol) followed by heating at 120℃ for 10 min. To estimate the relative amount of pullulan in crude exopolysaccharides, thin layer chromatograms were scanned with TLC scanner (Fiber Optic Scanner, KONTES).

Light microscopy

During the growth in liquid medium, samples were taken at intervals and photographed directly using a Spencer phase contrast light microscope (American Optical Cooperation) equipped with polaroid camera.

Results and Discussion

Effect of initial pH on the cell growth and the exopolysaccharide production

The effects of initial pH of culture broth on the cell growth and the exopolysaccharide production were examined. As shown in Fig. 1, the maximum cell growth, 35 g/l, was obtained at the initial pH 3.0 but at this pH, the exopolysaccharide production was less than 4.0 g/l. The maximum exopolysaccharide production, 22.5 g/l, was obtained at the initial pH 7.5. This result indicated that the optimal pH for the exopolysaccharide production is different from that of the cell growth. However, the optimal pH of the cell growth and exopolysaccharide production may not be coincided with the above results, because the pH culture broth changed during the cultivation.

Effect of controlled pH on the cell growth and the exopolysaccharide production

A. pullulans was cultivated in jar fermentor containing 3 l of medium under the controlled pH and the results are shown in Fig. 2. When cells were cultivated on 5% sucrose medium containing 0.1% yeast extract, the total dry cell weights were very similar irrespective of the experimental pHs of culture broth. However, remarkable differences were observed in the exopolysaccharide production. At the constant pH 7.5 and 2.5, the exopolysaccharide productions were very low, 6.5 and 6.0 g/l, respectively. At the constant pH 4.5, the maximum exopolysaccharide was obtained, about 24.5 g/l. From the results of Fig. 1 and 2, the cell growth seemed to be maximum at pH 2.5~3.0, but exopolysaccharide production seemed to be maximum at pH 4.5. However, when the initial pH 7.5 of culture broth was allowed to decrease spontaneously to pH 4.5 without pH control, the production of exopolysaccharide was 27.5 g/l, which was slightly higher than that of the constant pH 4.5 with pH control. This result was considered to be obtained from the combined effects of the pH of culture broth and the morphology of *A. pullulans*, which will be discussed in late. Since *A. pullulans* shows dimorphic growth, we examined the effect of constant pHs on the morphology of *A. pullulans*. As shown in Fig. 3, at the constant pH 2.5, most of the initial yeast-like cells transformed into the mycelial form at the exponential phase of the growth, and with the increase of the cultivation time, the elongation of mycelium was occurred and finally they were cross-linked with each other, resulting in the pellet shape. At this morphological state, the exopolysaccharide production was very low. While at the constant pH 7.5, the cells grew exponentially by budding, just like the yeast, *Saccharomyces cerevisiae* and at the statio-

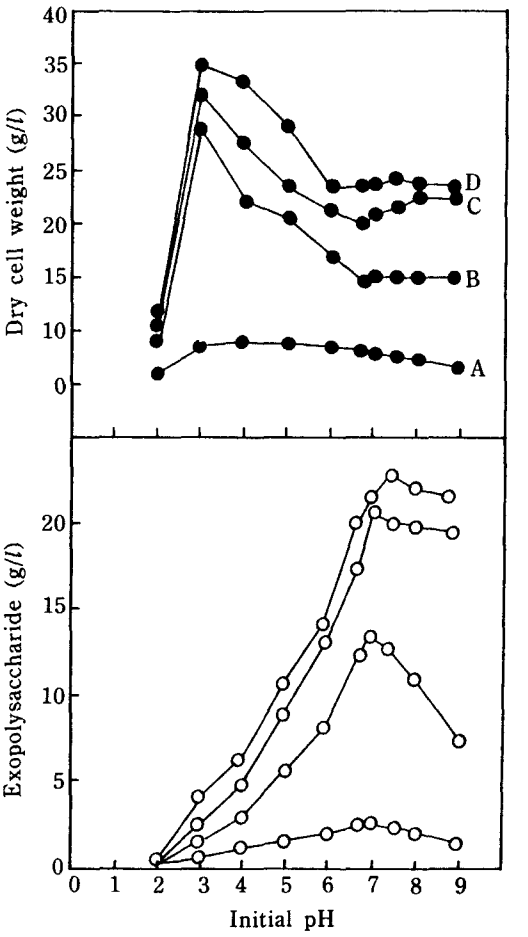

Fig. 1. Effects of the initial pHs of culture broth on the cell growth and exopolysaccharide production. Experiments were conducted for 500 ml Erlenmeyer flasks containing 100 ml of medium (10% gucose+0.4% yeast extract+salt mixture). Dry cell weight (●), exopolysaccharide (○), and A, B, C, and D represent the sampling time of 1, 3, 5, and 7 days, respectively.

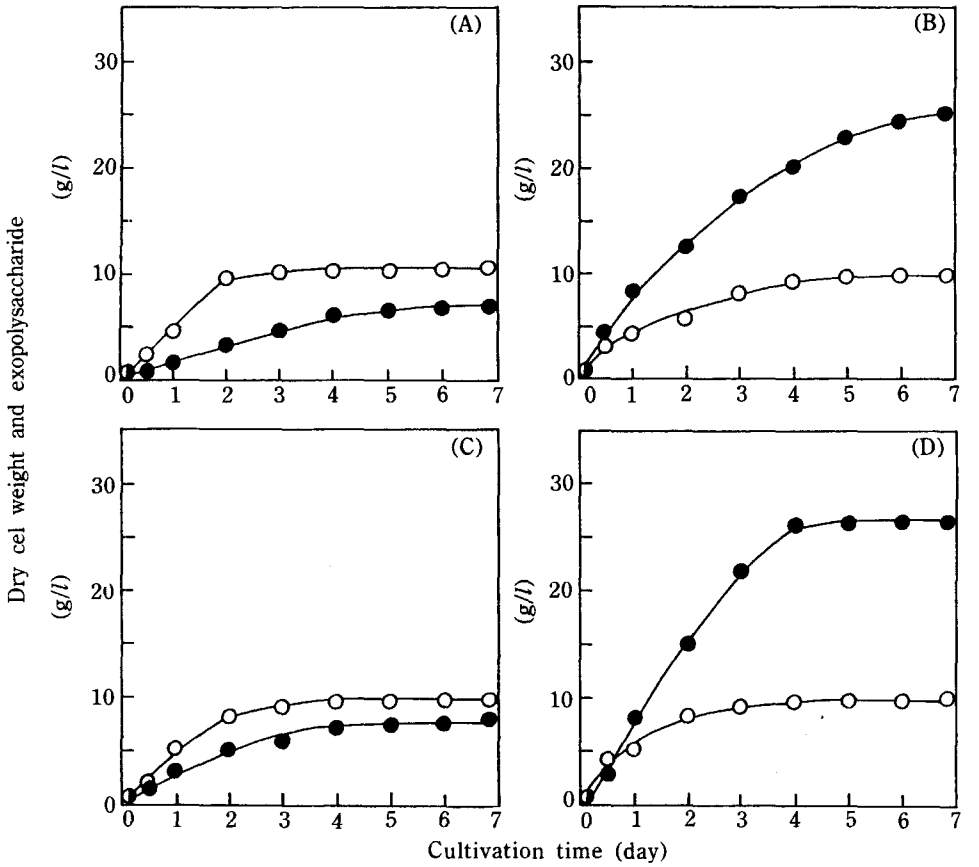

Fig. 2. Effects of constant pH of culture broth on the cell growth and exopolysaccharide production.
Experiments were conducted for jar fermenter containing 3 *l* of medium (5% sucrose+0.1% yeast extract+salt mixture) at constant pH 2.5 (A), constant pH 4.5 (B), constant pH 7.5 (C), and initial pH 7.5 without pH control (D). Dry cell weight (○) and exopolysaccharide (●).

nary phase, most of the cells were yeast-like form in their morphology. With the increase of the cultivation time, the yeast-like cells were changed into the large swollen cells and showed blackness around their cells walls. At this morphological state, the exopolysaccharide production was also very low. At the constant pH 4.5, both the mycelial and yeast-like forms were observed. At this morphological state, the exopolysaccharide production was high. Because the morphology of cells was distinctive at each constant pH, the exopolysaccharide production seemed to be affected by the morphology of the cells and the pH of culture broth.

Effect of pH shift on the exopolysaccharide production

The pH shift experiments were carried out and the transitional changes were analyzed for the acquirement of the more informations on the effect of pH and on the morphology of cells. Fig. 4 represents the pH shift from pH 2.5 to pH 4.5. After the shift of pH at the 2nd day of cultivation, the exopolysaccharide production sharply increased without lag. This results indicated that the mycelial form of cells cultivated at constant pH 2.5 contained the sufficient ability to synthesize the exopolysaccharide at pH 4.5. At this pH shift, the maximum specific production rate of exopolysaccharide was about 0.048 g product/g cell/hr. When the pH was shifted from the constant pH 7.5 to pH 4.5, as shown in Fig. 5, the exopolysaccharide production was also prompted to increase without lag. This

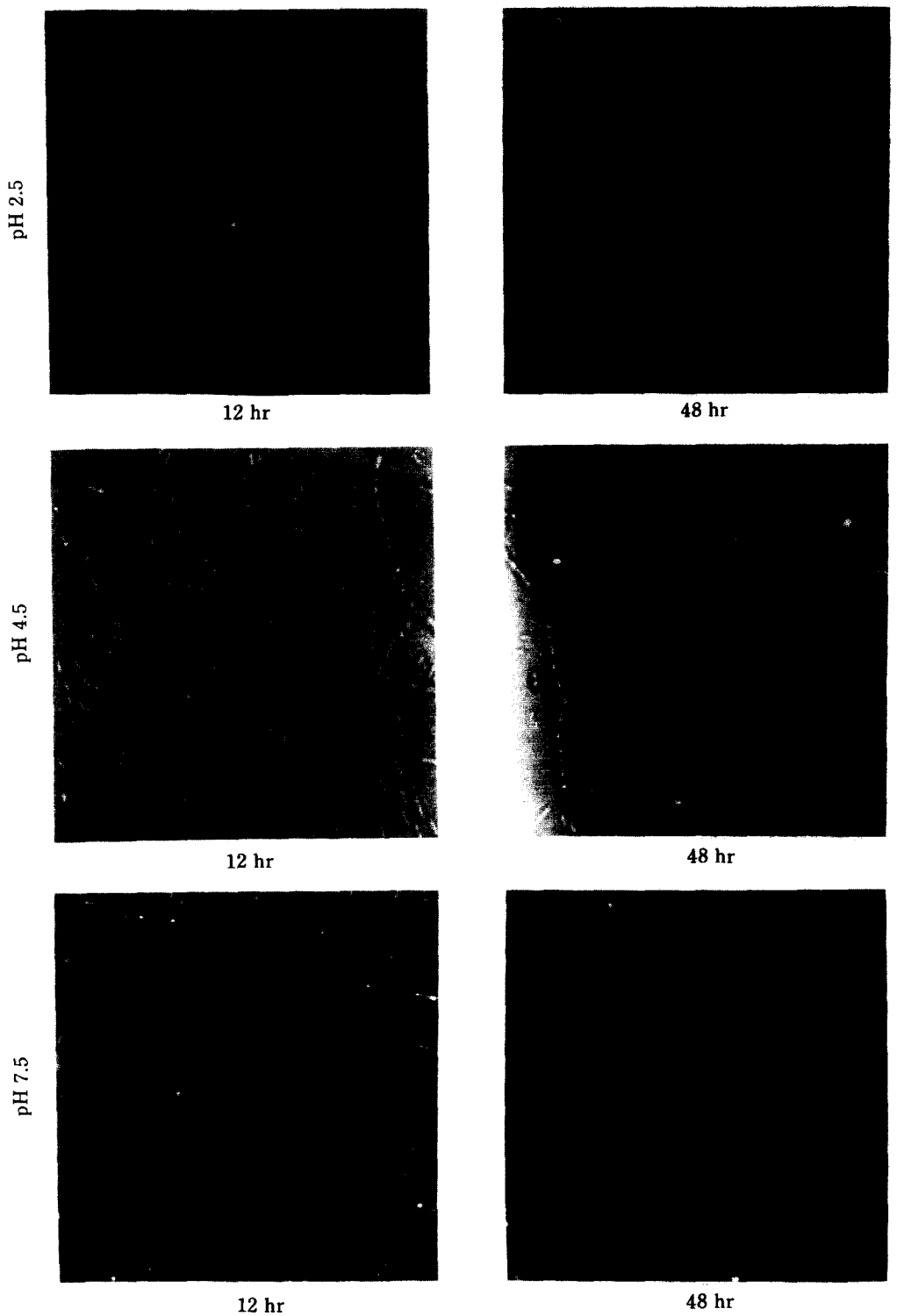

Fig. 3. Photographs of *A. pullulans* observed at different pHs.

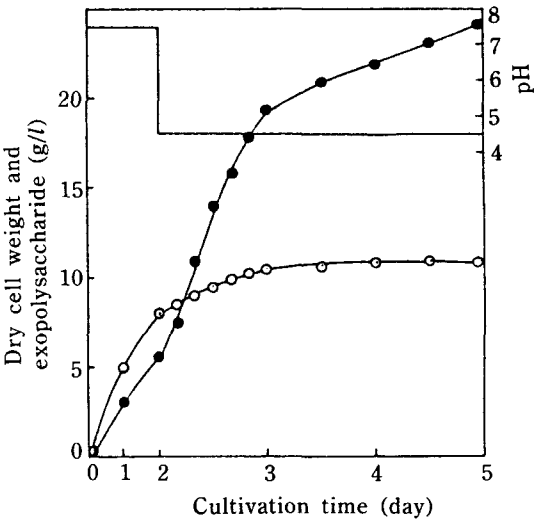

Fig. 4. Effect of pH shift from pH 7.5 to pH 4.5 on the exopolysaccharide production.
Dry cell weight (○), exopolysaccharide (●), and pH(−).

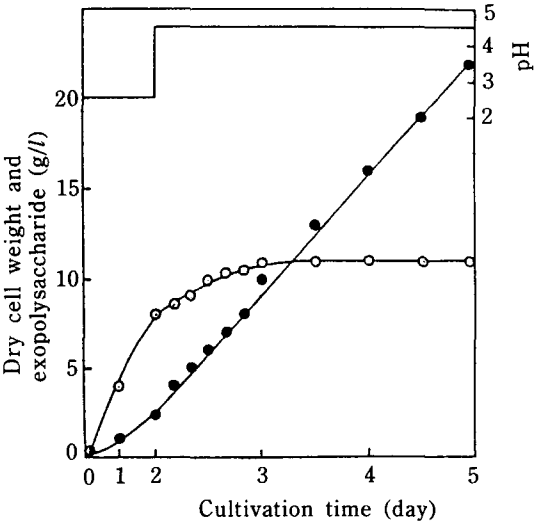

Fig. 5. Effect of pH shift from pH 2.5 to pH 4.5 on the exopolysaccharide production.
Dry cell weight (○), exopolysaccharide (●), and pH(−).

shows that the yeast-like form of cells cna also elaborate the exopolysaccharide efficiently at pH 4.5. At this pH shift, the maximum specific production rate of exopolysaccharide was 0.058 g product/g cell/hr. From these results, it is concluded that the yeast-like form of *A. pullulans* shows the slightly higher production rate of exopolysaccharide than the mycelial form and these two forms of *A. pullulans* can efficiently synthesize exopolysaccharide at pH 4.5.

In summary, the pH of culture broth was very important for the cell growth, exopolysaccharide production, and the morphological change of cells. At constant pH 7.5, most of cells were yeast-like form and at constant pH 2.5, most of cells were mycelial form. During the cultivation of cells with controlled pHs, these two forms of cells yielded small amount of exopolysaccharide. But relatively high exopolysaccharide production was obtained at the constant pH 4.5. The exopolysaccharide synthesis seemed to be optimal around pH 4.5. From the pH shift experiments, the mycelial form of cells cultivated at constant pH 2.5 and the yeast-like form of cells cultivated at the constant pH 7.5 also successfully elaborated the exopolysaccharide after the shift of pHs to pH 4.5, which implied that the mycelial form and yeast-like form of *A. pullulans* contains the sufficient exopolysaccharide synthesis system but when the environmental pHs are not optimal for the exopolysaccharide synthesis, these two forms of cells can not efficiently elaborate the exopolysaccharide. Previous investigators reported that the yeast-like form is major one in the exopolysaccharide production (13, 20) or the morphological change from initial mycelial form to yeast-like form may enhance the exopolysaccharide formation (5). In our research, it was proved that the yeast-like form and mycelial form of *A. pullulans* contains the similar biosynthetic activities of exopolysaccharide and the yeast-like form shows only slightly the higher ability to synthesize the exopolysaccharide than the mycelial form. The important factor for the production of exopolysaccharide is not just the morphology of cells but the pH of culture broth which affects the overall metabolic activities of *A. pullulans*.

Analysis of exopolysaccharide

For the identification and quantification of pullulan in the crude exopolysaccharide. TLC was carried out. When the crude exopolysaccharide was hydrolized with pullulanase, only maltotriose was detected as shown in Fig. 6, which indicated that the crude exopolysaccharide is pullulan. By scan-

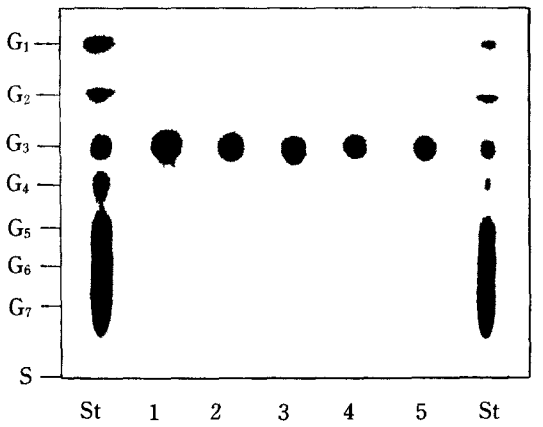

Fig. 6. Identification of pullulan by thin layer chromatography (TLC).
Lane St: standard sugrs, lanes 1 and 5: authentic pullulan treated with pullulanase, lanes 2, 3 and 4; exopolysaccharides taken at the 2nd, 5th, and 7th day of cultivation and treated with pullulanase. G represents the degree of polymerization of glucose.

ning the TLC plate with densitometer, it was observed that higher than 90% was pullulan in the crude exopolysaccharides extract.

요 약

불완전 곰팡이의 일종인 *Aureobasidium pullulans* IFO 4464 균주를 이용하여 배지의 pH가 풀루란의 생성 및 균의 형태에 미치는 영향을 조사하였다. pH 2.5에서는 균사형태, pH 7.5에서는 효모형태의 성장을 하였으며, 이 두 pH 조건하에서는 모두 낮은 풀루란 생산성을 보였다. 그러나 pH 4.5 조건에서는 균사형태와 효모형태가 혼합된 성장을 보였으며, 이 때 최고의 풀루란 생산을 나타냈다. pH 2.5에서 성장한 균사형태나, pH 7.5에서 성장한 효모형태를 배지의 pH를 4.5로 바꾸고 배양하였을 때 모두 풀루란 생산이 급격히 증가하였으며, 이 때 최대 비생산속도가 각각 0.048(hr^{-1}), 0.058(hr^{-1})이었다.

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