*Journal of Microbiology and Biotechnology*
Vol. 3, No. 4, 298-302, 1993

NOTE

# Pullulan Production from Starch Hydrolysate by *Aureobasidium pullulans* SH8646

**SHIN, YONG-CHUL[1]*, TAE-UN KIM[2] AND SI-MYUNG BYUN[3]**

*[1]Department of Microbiology, Gyeongsang National University, Chinju 660-701,*
*[2]Department of Clinical Pathology, Jisan Junior College, Pusan 609-323,*
*[3]Department of Life Science, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea*

**Pullulan was produced from starch hydrolysate with *Aureobasidium pullulans* SH8646. We could measure the correct amount of pullulan produced without the interference of starch from the culture supernatant by using a bacterial α-amylase treatment and ethanol: acetone (1 : 1) precipitation. When 5% acid-hydrolyzed starch was used as a carbon source, the dry cell weights obtained were similar irrespective of DE values of starch hydrolysates. The dry cell weights of those on the starch hydrolysate media prepared with 0.1 N HCl treatment, were slightly higher (9.5~10.5 g/l) than those on the starch hydrolysate media prepared with 1.0 N HCl (8.5~9.5 g/l). And among the starch hydrolysates showing DE values lower than 50, maximum pullulan production of 15 g/l was obtained at DE 30~40 starch hydrolysate but those showing DE values higher than 50, the pullulan production was increased with the increase of the DE value of starch hydrolysates. From the media containing 5%, 10%, and 15% starch hydrolysate (DE 25, 45, and 75), about 20~34% pullulan yield was obtained and the maximum pullulan yield of 34% (17g/l) was obtained from 5% DE 75 starch hydrolysate. The pullulan yields from starch hydrolysate media were much lower than those from glucose, maltose, maltotriose, and sucrose media.**

Pullulan is an extracellular polysaccharide produced from the yeast-like fungus *Aureobasidium pullulans* and a linear chain of maltotriose units in α-(1, 6) linkage (2, 4, 14, 15). Pullulan can be applied in food, cosmetic, and other industries as oxygen-impermeable coatings, adhesives, and viscosity enhancer (16, 18) and also can be used as a dielectric material in the forms of cyanoethyl-pullulan (10). In the near future, its greatest use may be as a biodegradable plastic substituting the petroleum-based products which are not biodegradable.

It has been reported that *A. pullulans* can produce pullulan from glucose, fructose, xylose, galactose, arabinose, rhamnose, sucrose, maltose, lactose, cheese whey, inulin, peat hydrolysate, and starch hydrolysate (1, 3, 6, 8, 12, 13, 16, 17). Among the carbon sources examined until to date, the highest pullulan yield, about 76% (70 g/l), was obtained from 10% partial hydrolysate of starch, dextrose equivalent (DE) 50 (16). In spite of this high pullulan yield, detailed experimental results have not yet been reported and there is some controversy surrounding this result. Shipman and Fan (13) tried to utilize starch syrup for the production of pullulan and a single cell protein. However, they used starch syrup, mainly composed of glucose after the complete enzymatic or acid hydrolysis, which is quite different from the partial hydrolysate of starch. A recent paper (7) reported that the color variant strains of *A. pullulans* made significant amount of pullulan from the unconventional lactose, xylose, and starch substrates. However, the yield of polysaccharide from these carbon sources is lower than 30% and as much as 70 to 80% of this polysaccharide was not pullulan.

When we tried to utilize the starch hydrolysate for the production of pullulan with *A. pullulans*, the first problem to be solved was the correct measurement of pullulan from the culture supernatant because the amount of pullulan was overestimated by co-precipitation of starch hydrolysate present in culture broth by conventional solvent precipitation method. This paper is the first in investigating the pullulan production from partially hydrolyzed starch medium by using the correct measurement of pullulan produced without the interferene of starch in the culture supernatant.

*Corresponding author
Key words: *Aureobasidium pullulans* SH8646, pullulan, starch hydrolysate

## MATERIALS AND METHODS

### Cultivation of Microorganism

*A. pullulans* SH 8646 is a mutant strain derived from *A. pullulans* IFO 4464 with the treatment of a nitrosoguanidine mutagen. The maintenance and preparation of inoculum were the same as described in our previous paper (12). For the utilization of starch hydrolysate, the medium was prepared by substituting starch hydrolysate for sucrose and subtracting NaCl in AYS medium (12). All of the experiments were conducted in 500 m*l* Erlenmeyer flasks containing 100 m*l* medium at 27℃, 200 rpm with a shaking incubator.

### Acid Hydrolysis of Starch

10 g of potato starch (Shimakyu Chem. Co.) was dissolved in 50 m*l* of HCl solution (0.1 N or 1.0 N) and hydrolyzed in the boiling water bath for various time intervals. The starch hydrolysate was removed from the boiling water bath and rapidly cooled on 4℃ and then, neutralized to pH 7.0 with a concentrated NaOH solution. The amount of reducing sugar in the starch hydrolysate was determined by dinitrosalicylic acid method (9). The degree of acid hydrolysis was expressed as dextrose equivalent (DE):

$$DE(\%) = \frac{\text{reducing sugar of sample measured as glucose(g)}}{\text{sample dry weight(g)}} \times 100$$

### Measurement of Dry Cell Weight and Exopolysaccharide

When starch hydrolysate was used as a carbon source, 2 m*l* of supernatant of culture broth was treated with 43 units of a saccharogenic bacterial α-amylase (*Bacillus* sp., A3051, Sigma Chemical Co.) for 4 hr at 37℃ and then exopolysaccharide was precipitated with one volume of ethanol: acetone (1 : 1) solution. The precipitated exopolysaccharide was washed twice with ethanol and acetone and dried to a constant weight in an oven at 105℃. Dry cell weight was measured as described in our previous paper (12).

### Sugar Composition of Starch Hydrolysate

After the filtration of starch hydrolysate with a 0.45 μm Millipore filter, the sugar compositions of starch hydrolysate were analyzed with a HPLC system (Waters Associates) equipped with a Lichrosorb $NH_2$ (10 μm) column (Merck, Germany). The solvent was acetonitrile and water (73 : 27), and the flow rate was 1.8 ml/min. Sugar peaks were detected with a differential refractomer. To measure the amount of each sugar, standard curves were used for glucose(G1), maltose(G2), maltotriose(G3), maltotetraose(G4), maltopentaose(G5), and maltohexaose(G6).

### Determination of Hydroxymethyl Furfural (HMF)

The concentration of HMF present in the starch hydrolysate was determined by absorbance measurements at 285 nm using standard aqueous solutions of this compound (Aldrich Chemical Co., Inc.) (5). The data herein are expressed as HMF mg/100 g dry weight of sample.

## RESULTS AND DISCUSSION

### Measurement of Pullulan on Soluble Starch Medium

The use of starch hydrolysate as a carbon souce evokes a problem in the correct measurement of pullulan because the starch hydrolysate remaining in culture medium are co-precipitated by a conventional solvent precipitation method with the pullulan produced from *A. pullulans*. Therefore, the quantity of pullulan measured is overestimated because of the contamination of the co-precipitated starch hydrolysate. To solve this problem, a selective enzymatic hydrolys is of the starch hydrolysate in fermentation broth was tried. As shown in Fig. 1, pullulan was not attacked by the saccharogenic bacterial α-amylase from *Bacillus* sp., but the soluble starch was rapidly hydrolyzed by this enzyme and yielded unprecipitatable sugars. By the method of bacterial α-amylase treatment and the solvent precipitation, the pullulan mixed with the soluble starch could be accurately measured within 5% error range without the interference of the soluble starch.

### Preparation of Starch Hydrolysates

Because the starch solution is very viscous and needs to be hydrolyzed to utilizable sugars by cells before absorption, it is convenient and effective for utilizing as carbon source to hydrolyze starch to a certain degree of polymerization. The starch hydrolysates were prepared by the treatment of HCl solutions. As shown in Fig. 2, 1.0 N HCl rapidly hydrolyzed starch and within 25 min, a starch hydrolysate of DE 90 was obtained. But 1.0 N HCl produced a lot of hydroxymethyl furfural (HMF) during acid hydrolysis, which is considered as a potential inhibitor of the microbial growth. 0.1 N HCl solution hydrolyzed starch slowly and produced a very small amount of HMF. Table 1 shows the composition of sugars in the starch hydrolysates obtained at various DE values. At DE 75, most of the carbohydrates were G1, G2, G3, and G4 maltooligosaccharides lower than G6. At DE 30, 55.8% of carbohydrates was maltooligosaccharides higher than G6.

### Pullulan Production from Starch Hydrolysates

The starch hydrolysates prepared by the treatment of 0.1 N or 1.0 N HCl solutions were used as carbon sources for the production of pullulan. Fig. 3 shows the

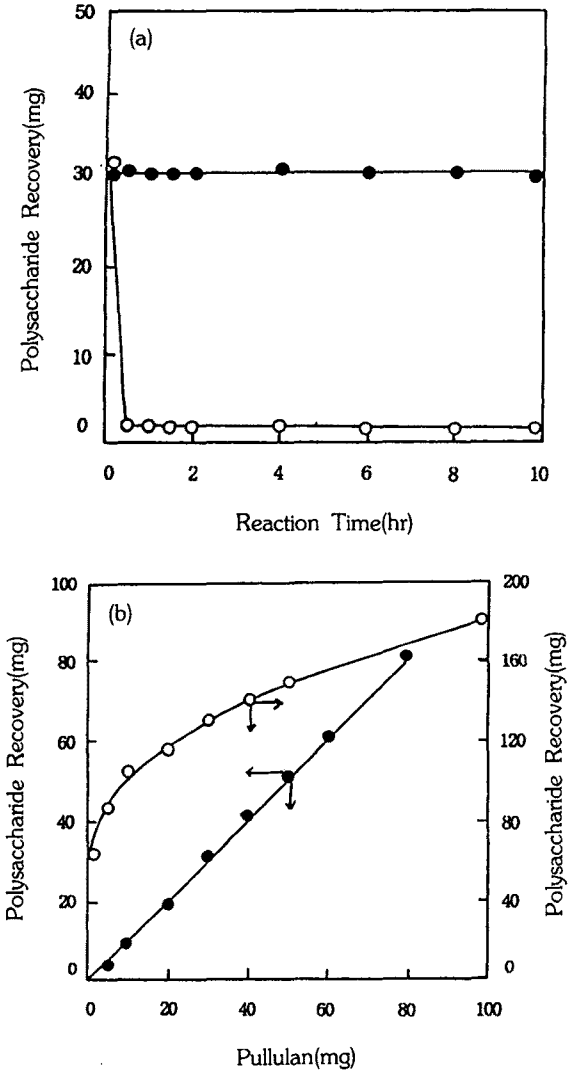


**Figure 1. Selective measurement of pullulan from starch-contaminated solution by a bacterial α-amylase treatment and solvent precipitation.**

(a) Effect of *Bacillus* sp. α-amylase on the hydrolysis of soluble starch and pullulan. 50 mg of soluble starch (○) and 30 mg of pullulan (●) were treated with 100 units of the enzyme at 37℃ for various time intervals and the remaining polysaccharides was recovered by solvent precipitation. (b) Selective measurement of pullulan from starch-contaminated solution. The various amounts of pullulan were added to 100 mg of soluble starch and the mixtures were treated with 100 units of the amylase, and remaining polysaccharides were recovered by the solvent precipitation (●). Symbol ○ represents the polysaccharides obtained by solvent precipitation without the treatment of α-amylase.

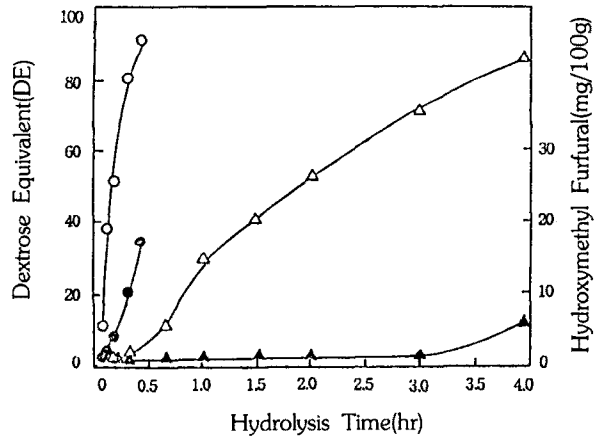


**Fig. 2 Acid-hydrolysis of potato starch.**

20% potato starch solution was hydrolyzed by 1.0 N HCl solution (circle) or 0.1N HCl solution (triangle) in the boiling water bath. Dextrose equivalent, ○ and △; hydroxymethylfurfural, ● and ▲.

**Table 1. Sugar compositions of starch hydrolysates prepared by 0.1 N HCl treatment**

| Sugar | DE 30 | DE 45 | DE 55 | DE 75 |
|---|---|---|---|---|
| G1 | 8.0 | 14.7 | 20.2 | 33.5 |
| G2 | 9.3 | 14.5 | 16.5 | 24.1 |
| G3 | 7.8 | 12.5 | 12.3 | 17.4 |
| G4 | 7.7 | 10.5 | 9.8 | 12.6 |
| G5 | 6.9 | 9.2 | 8.3 | 7.3 |
| G6 | 4.5 | 9.1 | 6.8 | 5.3 |
| Higher oligomers | 55.8 | 29.8 | 26.1 | 0 |

G1, G2, G3, G4, G5, and G6 represent glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose, respectively. DE represents dextrose equivalent.

effect of DE values on the cell growth and exopolysaccharide production. Dry cell weights obtained were nearly the same, irrespective of the DE values of starch hydrolysate but there were some differences in the dry cell weights by the preparation methods of starch hydrolysate. Starch hydrolysates prepared by 0.1 N HCl treatment showed higher cell growth, about 9.5~10.5 g/*l*, than those prepared by 1.0 N HCl showing 8.5~9.5 g/*l*. When the starch hydrolysates showing DE values lower than DE 50 were used as carbon sources, the maximum pullulan production was obtained at DE 30~40. However, when the starch hydrolysates showing DE values higher than DE 50 were used, the pullulan production was increased with the increase of DE value of starch hydrolysate. When the production of pullulan was investigated at various concentrations of starch hydrolysates, the highest conversion yield (Yp/s), 34% (17 g/*l*), was obtained from 5% of DE 75 starch hydrolysate and with the increase of the starch hydrolysate concentration, pullulan yield was inversely decreased (Table 2).

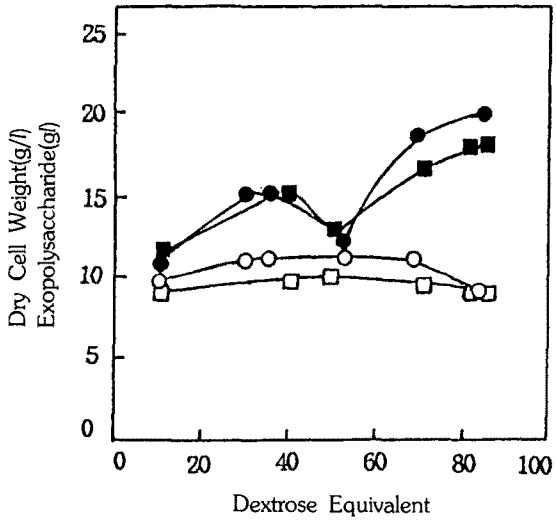


**Fig. 3. Effect of dextrose equivalent on the cell growth and exopolysaccharide production.**
Starch hydrolysates were prepared by the treatment of 0.1 N HCl (circle) or 1.0 N HCl (square). The 7 days-cultures were analyzed for the measurement of dry cell weight (○, □) and exopolysaccharide (●, ■).

**Table 2. Production of pullulan from starch hydrolysate by *A. pullulans* SH8646**

| Dextrose equivalent | Concentration (%) | Pullulan (g/l) | Pullulan yield (%) |
|---|---|---|---|
| 25 | 5 | 15 | 30 |
| | 10 | 25 | 25 |
| | 15 | 35 | 23 |
| 45 | 5 | 12 | 24 |
| | 10 | 20 | 20 |
| | 15 | 30 | 20 |
| 75 | 5 | 17 | 34 |
| | 10 | 30 | 30 |
| | 15 | 35 | 23 |

Pullulan yield represents the percent ratio of pullulan produced to substrate added. Pullulan content was determined after 7-days cultivation.

The growth of *A. pullulans* on the starch hydrolysate medium was similar to those grown on the mono- or disaccharides medium (11), but the pullulan production from starch hydrolysate was much lower than those from on mono- or disaccharides. The lower pullulan yield from starch hydrolysate was strongly supported by the results of Table 3 indicating that with the increase of the chain length of glucose unit, the pullulan production was inversely decreased. The pullulan yield obtained in this study

**Table 3. Effects of the chain length of glucose unit on pullulan production**

| Carbon source | Cell growth (g/l) | Pullulan (g/l) | Pullulan yield (%) |
|---|---|---|---|
| Glucose | 9.5 | 22.5 | 45 |
| Maltose | 9.0 | 19.0 | 38 |
| Maltotriose | 9.0 | 18.0 | 36 |
| Soluble starch | 8.5 | 12.5 | 25 |

5% of each carbon source was added to AYS medium, and the dry cell weight and pullulan content were determined after 7-days cultivation.

was about one third or a half of that of Yuen (16) who obtained up to 76% pullulan yield (76 g/l) of the starch hydrolysate (DE 50) with *A. pullulans* IFO4464. Leathers *et al.* (7) reported that only 21% pullulan yield was obtained from starch by *A. pullulans* IFO 4464. Although further research is required to explain the difference between our results and Yuen's (16), our results lead us to suspect that the yield reported by Yuen may be erroneous, which probably occurred when measuring the pullulan in starch hydrolysate medium.

## Acknowledgement

This study was supported by a research grant from Korea Science and Engineering Foundation (Project No. 91-05-00-17).

## REFERENCES

1. **Boa, J.M. and A. Leduy.** 1984. Peat hydrolysate medium optimization for pullulan production. *Appl. Environ. Microbiol.* **48**: 26-30.
2. **Bouveng,H.O., H. Kiessling, B. Lindberg, and J. McKay.** 1962. Polysaccharides elaborated by *Pullularia pullulans*. **PartII.** The partial acid hydrolysis of the neutral glucan synthesized from sucrose solutions. *Acta Chem. Scand.* **17**: 797-800.
3. **Catley, B.J.** 1971. Utilization of carbon sources of *Pullularia pullulans* for the elaboration of extracellular polysaccharides. *Appl. Microbiol.* **22**: 641-649.
4. **Catley, B.J. and W.J. Whelan.** 1971. Observations on the structure of pullulan. *Arch. Biochem. Biophys.* **143**: 138-142.
5. **Haas, V.A., E.R. Stadtman, F.H. Stadtman and G. Mackinney.** 1948. Deterioration of dried fruits. I. The effect of sugars and furfurals. *J. Am. Chem. Soc.* **70**: 3576-3579.
6. **Kato, K. and M. Shiosaka.** 1975. Process for the production of pullulan. U.S. Patent 3,912,591.
7. **Leathers, T.D., G.W. Nofsinger, C.P. Kurtzman, and R.J. Bothast.** 1988. Pullulan production by color variant strains

of *Aureobasidium pullulans*. *J. Ind. Microbiol.* **3**: 231-239.
8. **LeDuy, A., J.J. Yarmoff, and A. Chagraoui.** 1983. Enhanced production of pullulan from latose by adaptation and by mixed culture techniques. *Biotechnol. Lett.* **5**: 49-54.
9. **Miller, G.L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426-428.
10. **Onda, Y., H. Muto, and H. Suzuki. March** 1982. Cyanoethyl pullulan. U.S. patent 4,322,524.
11. **Shin, Y.C., J.K. Han, Y.H. Kim, H.S. Lee, and S.M. Byun.** 1987. Inhibition effect of sugar concentrations on the cell growth and the pullulan production of *Aureobasidium pullulans*. *Kor. Jour. Microbiol.* **25**: 360-366.
12. **Shin, Y.C., Y.H. Kim, H.S. Lee, S.J. Cho, and S.M. Byun.** 1989. Production of exopolysaccharide pullulan from inulin by a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis*. *Biotechnol. Bioeng.* **33**: 129-133.
13. **Shipman, R.H. and L.T. Fan.** 1978. Bio-plastics and SCP from starch and agricultural wastes. *Process Biochem.* **13**: 19-21.
14. **Sowa, W., A.C. Blackwood, and G.A. Adams.** 1963. Neutral extracellular glucan of *pullularia pullulans* (De Bary) Berkhout. *Can. J. Chem.* **41**: 2314-2319.
15. **Wallenfels, K., G. Bechtler, and D. Freudenberger.** 1965. Untersuchungen an pullulan. 4. Die Klarung des strukturproblems mit physikalischem, chemischen, und enzymatischen methoden. *Biochem. Z.* **341**: 433-450.
16. **Yuen. S.** 1974. Pullulan and its applications. *Process Biochem.* **9**(11): 7-9, 22.
17. **Zajic, J.E.** 1967. Process for preparing a ploysaccharide flacculating agent. U.S. Patent 3,320,136.
18. **Zajic, J.E. and A. LeDuy.** 1973. Flocculant and chemical properties of a polysaccharide from *Pullularia pullulans*. *Appl. Microbiol.* **25**: 628-635.

**(Received August 26, 1993)**