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Short communication

Influence of controlled pH on the activity of UDPG-pyrophosphorylase in *Aureobasidium pullulans*

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ABSTRACT

UDPG-pyrophosphorylase is the key enzyme involved in pullulan biosynthesis and pullulan production by *Aureobasidium pullulans*. In this study, effect of controlled pH on fermentation time, pullulan production, biomass, and UDPG-pyrophosphorylase activity was investigated. Pullulan yield increased to reach a maximum within 4 days, and maximum UDPG-pyrophosphorylase activity was observed at day 3, while the biomass continued to increase until the end of the experimental period. The *A. pullulans* isolated from sea mud grew well at relatively low pH. UDPG-pyrophosphorylase activity was affected by the controlled pH and reached a maximum at pH 5.5. Results indicated that UDPG-pyrophosphorylase activity was highly correlated with controlled pH and pullulan biosynthesis rate.

1. Introduction

Pullulan, an exocellular homopolysaccharide of economic importance produced by the yeast-like fungus *Aureobasidium pullulans*, is composed of maltotriosyl units linked through α-(1→6) glycosidic bonds. It is used as a coating and in packaging materials, as a sizing agent for paper, a starch replacement in low-calorie food formulations, in cosmetic emulsions, and in other industrial applications since it is resistant to oils and grease, non-toxic, impermeable to oxygen, and least affected by temperature (Singh, Saini, & Kennedy, 2008).

As a precursor for pullulan synthesis in *A. pullulans*, UDP-glucose is essential for the biosynthesis of pullulan by *A. pullulans* (Shingel, 2004). It is formed from UTP and glucose 1-phosphate in reverse reaction catalyzed by UDPG-pyrophosphorylase: UTP + glucose-1-phosphate → UDP-glucose + PPi (Daran, Dallies, Thines-Sempoux, Paquet, & Francois, 1995). Therefore, the activity of UDPG-pyrophosphorylase should be of importance in pullulan production and was confirmed by Duan et al.: the activity of UDPG-pyrophosphorylase was highly correlated with the amount of pullulan produced and the carbon source used (2008). However, the relationships among pH, UDPG-pyrophosphorylase activity, and pullulan production have not been studied.

Therefore, it is of our interest to investigate the effect of controlled pH and fermentation time on pullulan production, biomass accumulation, UDPG-pyrophosphorylase activity, and morphology of *A. pullulans* CJ001 isolated from sea mud.

2. Materials and methods

2.1. Microorganism

A. pullulans CJ001 isolated from sea mud was obtained from senior Technician Jing Chen, School of Marine Science and Technology, HuaiHai Institute of Technology, China. Stock cultures were maintained on potato dextrose agar at 4 °C and subcultured every 2 weeks.

2.2. Preparation of inoculum medium

The medium contained 50 g sucrose, 2.0 g yeast extract, 0.77 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1.98 g NaCl in 1 L distilled water. The pH was adjusted to 5.5, and the medium was autoclaved at 121 °C for 15 min (Chen, Wu, & Pan, 2012).

2.3. Fermentation

Fermentation was conducted according to Jiang et al. with slight modifications (2011). Seed culture was prepared by inoculating cells grown on a potato dextrose agar slant into a 250-mL flask that contained 50 mL of the inoculum medium and subsequently incubated at 22 °C for 2 days with shaking at 200 rpm. A 5-L stirred tank fermentor (5M-2002, Shanghai Baoxing Bio-engineering Equipment Co., China) with a working volume of 3 L was used for the

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production of pullulan in batch culture. Fermentor was consisted of a glass vessel with stainless-steel endplates and three equally spaced vertical baffles. Agitation was provided by a six-flat-blade impeller (diameter 4 cm) located 3 cm above the bottom of the vessel. The fermentor was sterilized at 121 °C for 15 min. After cooling, 3 L of production medium was added into the fermentor. The medium was inoculated with 150 mL inoculum. The fermentor was incubated at 22 °C in a thermostated chamber (Wu, Chen, & Pan, 2012). The impeller speed was 800 rpm and the sterile air flow was 4 L/min. The pH was controlled at 5.5 by feeding with either 2 M NaOH or 2 M HCl.

2.4. Preparation of cell-free extract

The cells in 5.0 mL of the culture were collected by centrifugation at 8000 × *g* at 4 °C for 5 min, washed three times with ice-chilled distilled water and suspended in 1.0 mL ice-chilled 1.0 M Tris–HCl (pH 7.6) to make a thick paste. The product was homogenized in a Homogenizer (DY89-I, Xinzhi, Zhejiang, China) for 1 h on the ice bath. The cell debris was removed by centrifugation at 14,000 × *g* for 30 min at 4 °C. The supernatant was the cell-free extract (also the enzyme preparation). Protein concentration in the cell-free extract was determined by the method of Bradford with bovine serum albumin as standard (Duan, Chi, Wang, & Wang, 2008).

2.5. Enzymes assays

The reaction mixture contained 1.4 mM $NADP^+$, 4.0 mM $MgCl_2$, 1.0 mM UDP-glucose, 10 μM glucose 1,6-diphosphate, 66 mU of a-phosphoglucomutase, 220 mU of glucose 6-phosphate dehydrogenase, 75 mM Tris–HCl (pH 7.0), and 10 μM of a diluted enzyme preparation in a final volume of 0.5 mL. The absorbance was measured at 340 nm on a spectrophotometer using a 1 cm path cuvette at 30 °C. 1 unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μM of substrate in 1 min. The mixture with the diluted enzyme preparation that had been heated at 100 °C for 5 min was used as the control (Duan et al., 2008).

2.6. Isolation and purification of pullulan

The culture was centrifuged at 15,000 × *g* for 20 min to remove the microorganisms. After the supernatant was decanted, cells were weighed and then separated into unicellular and mycelial fractions using a 45 μm nylon mesh (Heald & Kristiansen, 1985). Weights of the unicellular and mycelial fractions were determined. An aliquot (3 mL) of the supernatant was transferred into a test tube, and then mixed thoroughly with 6 mL cold ethanol. The prepared mixture was left in a refrigerator (4 °C) for 12 h to precipitate the exocellular polysaccharide. Residual ethanol was removed carefully, then 3 mL deionized water was added and the mixture was heated to 80 °C in a water bath to dissolve the precipitate. The solution was dialyzed against deionized water for 48 h to remove small molecules. The polysaccharide was reprecipitated by adding 6 mL cold ethanol, and was recovered by filtering the mixture through pre-weighed Whatman GF/A filter paper. The filter paper with the recovered precipitate was dried at 80 °C to a constant weight (Badr-Eldin, El-Tayeb, El-Masry, Mohamad, & El-Rahman, 1994). The pullulan content of the ethanol precipitate was determined using the coupled-enzyme assay technique described by Israilides, Bocking, Smith, and Scanlon (1994). Cell biomass and pullulan content were expressed as g/L.

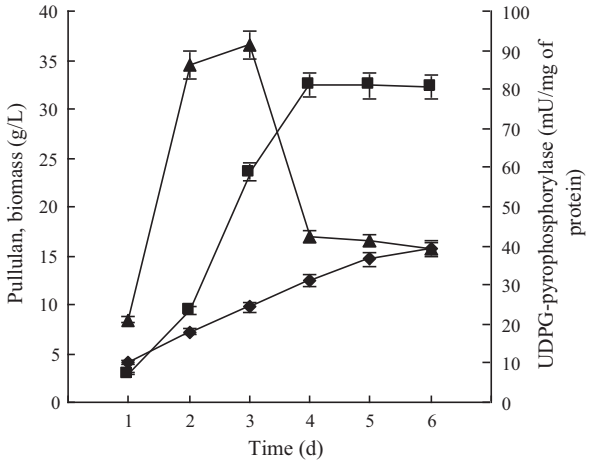

Fig. 1. Effect of time course on pullulan production (■), cell growth (♦), and UDPG-pyrophosphorylase (▲). Fermentation conditions: fermentation temperature, 22 °C; pH, 6.5. Data are shown as mean ± SD (n = 6). Bars represent the standard deviation.

2.7. Analytical methods

Pullulan molecular weight (M_W) was determined by High Performance Gel Filtration Chromatography (HPGFC) (LC-10A, Shimadzu, Japan) on a Ultrahydrogel Size Exclusion Column, which is capable of determining M_W in the range of 10^3–10^6. In the size exclusion chromatography studies, 0.1 N $NaNO_3$ was used as an eluent at a flow rate of 0.9 mL/min. The detector used was a High Sensitive Refractive Index Detector, Model ERC-7515 A (ERC Inc., Japan). The calibration of the detector was done with known concentrations of commercially available pullulan (Sigma). An aliquot of 20 μL was injected to the column after filtration through 0.45 μm millipore filter, at ambient temperature and the procedure was repeated three times. The software used was the Multi-channel Chromatography Data Station (Version 144A, 1993–1997 Ampersand Ltd.) (Jiang, Wu, & Kim, 2011). FT-IR spectrum of representative pullulan sample was collected in KBr pellets on a Nicolet Nexus FT-IR 470 spectrophotometer over a wavelength range of 400–4000 cm^{-1}.

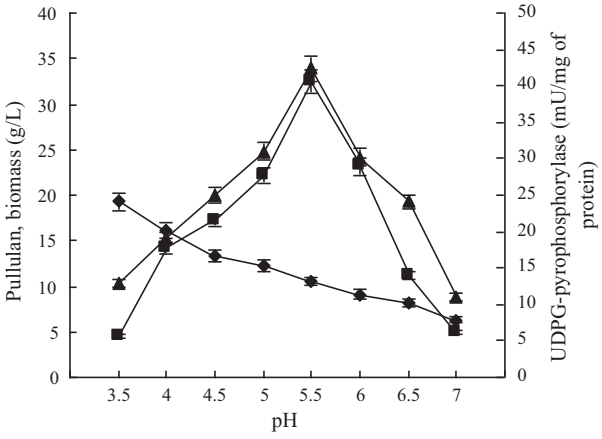

Fig. 2. Effect of pH on pullulan production (■)), cell growth (♦), and UDPG-pyrophosphorylase (▲). Fermentation conditions: fermentation temperature, 28 °C; fermentation time, 4 days. Data are shown as mean ± SD (n = 6). Bars represent the standard deviation.

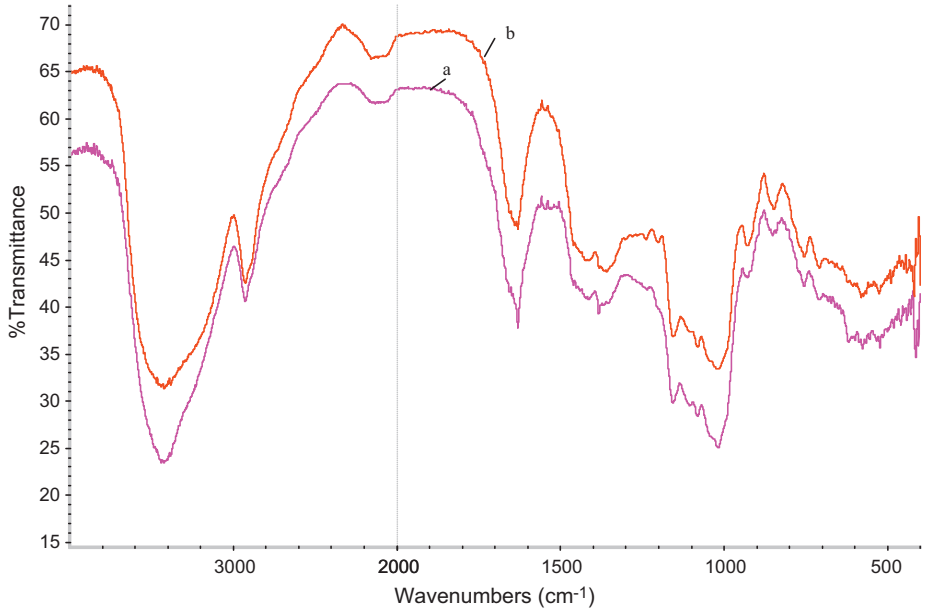

Fig. 3. FT-IR spectra of the pullulan obtained from Japan Pharmacopoeia (a) and the exopolysaccharide prepared in this experiment (b).

3. Results and discussion

3.1. Time course of fermentation

Time course of fermentation by *A. pullulans* CJ001 was made for a period of 6 days. Pullulan production increased sharply over 2–3 days, increased slightly from 3 days to 4 days and did not increase after 4 days (Fig. 1). The biomass increased steadily over the whole fermentation period (Fig. 1). So it is evident from the results that the production of pullulan is not associated with cell growth. The biomass was predominantly unicellular within 1 day, mainly mycelial at day 3, and almost entirely unicellular after day 4. This indicates that mycelial is responsible for pullulan production. This result is consistent with the previous reports (Cately, 1979; Heald & Kristiansen, 1985). The activity of UDPG-pyrophosphorylase reached maximum at day 3 and decreased sharply after day 4 (Fig. 1). Therefore, the activity of UDPG-pyrophosphorylase was highly correlated with pullulan biosynthesis rate, rather than the total amount of pullulan produced.

3.2. Effect of controlled pH on fermentation

Maximum pullulan production (32.47 g/L) and the highest activity of UDPG-pyrophosphorylase (75.21 mU/mg of protein) were observed at pH 5.5 (Fig. 2). Too low or too high pH decreased pullulan production and the highest activity of UDPG-pyrophosphorylase, even though biomass decreased with the increasing pH. In other reports, maximum pullulan production were 25.95 g/L (Vijayendra, Bansal, Prasad, & Nand, 2001), 25.1 g/L (Prasongsuk et al., 2007), and 30.28 g/L (Jiang, 2010), respectively. Yeast-like cell was observed at all the levels of pH, and relatively low pH is suitable for biomass growth and this observation is in agreement with those reported by Wu, Jin, Tong, and Chen (2009). Maximum UDPG-pyrophosphorylase activity was observed at pH 5.5, indicating that it is highly correlated with pH.

3.3. Characterization of the pullulan

The M_W of the representative exopolysaccharide prepared in this study is 3.1×10^5 Da, which is higher than that observed by Chen et al. (2012). This is may be ascribed to the different fermentation conditions. The FT-IR spectra of the exopolysaccharide prepared in this experiment and the pullulan obtained from Japan Pharmacopoeia are almost the same, indicating that the major composition of this exopolysaccharide is pullulan (Fig. 3) (Chen et al., 2012).

4. Conclusions

The influences of controlled pH on the pullulan production, cell growth, and activity of UDPG-pyrophosphorylase in the culture of *A. pullulans* CJ001 were studied. The maximum pullulan production was obtained on day 4 and at pH 5.5. Biomass continued to increase until the end of the experimental period (6 days). The activity of UDPG-pyrophosphorylase was highly correlated with pH and pullulan biosynthesis rate.

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