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Short communication

Effect of uracil on pullulan production by *Aureobasidium pullulans* CGMCC1234

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ABSTRACT

Effect of uracil on the pullulan production, biomass and uridine phosphorylase (UPase) activity was studied in this research. Uracil was found to enhance pullulan accumulation and the addition time of uracil was crucial to pullulan production. Pullulan yield of 49.07 g/L was achieved by adding 5 mM uracil at 48 h, by comparison to 37.72 g/L obtained with the control. UPase activity could not be detected at early growth stage of *Aureobasidium pullulans*, but stimulated by added uracil at logarithmic phase and stationary phase. The time course study on the fermentation of pullulan demonstrates that pullulan production was not closely associated with biomass accumulation. Results indicate that the increased pullulan yield brought by uracil was correlated with UPase activity.

1. Introduction

Pullulan, an exocellular homopolysaccharide, is produced by the strains of *Aureobasidium pullulans*. It is mainly made of maltotriose repeating units joined by (1 → 6)-α linkages (Pan, Yao, Chen, & Wu, 2013). It has a number of remarkable properties, such as nontoxicity, low viscosity, high plasticity and good film-forming. This polysaccharide is widely used in the food and pharmaceutical industries (Cheng, Demirci, & Catchmark, 2011).

Pullulan production was under complex metabolic and environmental control. It has been reported that UDP-glucose played key role in the biosynthesis of pullulan and could not be replaced by ADP-glucose, indicating that the pullulan chains and precursors originated from UDP-glucose (Shingel, 2004). As one of the nucleoside phosphorylases, UPase was an enzyme that participated in the pyrimidine salvage pathway of eukaryotes and prokaryotes (Ding et al., 2011). UPase, catalyzed the reversible phosphorolysis of the free pyrimidine base and ribose-1-phosphate to pyrimidine nucleosides, might be one of important enzyme in the pullulan biosynthesis in *A. pullulans*.

Addition of nucleotide bases was an essential factor in the polysaccharide synthesis in terms of metabolic driving force. For the synthesis of most of the polysaccharides, sugar nucleotides were served as the glycosyl donors. It had been found that the addition of nucleotide bases to the culture medium increased curdlan production by *Agrobacterium* species (West, 2006) and exopolysaccharide production by *Hericium erinaceus* (Lee, Wee, Lee, An, & Hong, 2010).

As far as we know, there were no reports recording the effect of nucleotide bases on the pullulan fermentation. The objectives of this study were to investigate the influence of uracil on pullulan production, biomass accumulation and UPase activity of *A. pullulans* CGMCC1234.

2. Materials and methods

2.1. Organisms and inoculum preparation

Non-pigmented strain of *A. pullulans* CGMCC1234 was maintained on potato dextrose agar (PDA) at 4 °C and subcultured every 2 weeks. The seed culture medium contained 50.0 g sucrose, 1.5 g yeast extract, 4.0 g K_2HPO_4, 0.8 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, and 2.0 g NaCl per liter of deionized water. The pH was adjusted to 6.5, and the medium was autoclaved at 121 °C for 15 min.

2.2. Fermentative production of pullulan

Cultures were grown on PDA at 28 °C for 4 days and then transferred to a 250-mL flask that contained 50 mL of the seed

Abbreviations: *A. pullulans*, *Aureobasidium pullulans*; UPase, uridine phosphorylase; ATP, adenosine triphosphate; UDP-glucose, uridine diphosphate glucose; ADP-glucose, adenosine diphosphate glucose; PDA, potato dextrose agar; DCW, dry cell weight.

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culture medium and subsequently incubated at 28 °C for 2 days with shaking at 200 rpm. The pullulan production was carried out in a 5 L stirred tank fermentor (KF-5l, KoBioTech Bio-engineering Equipment Co., Ltd.) with a working volume of 3 L of the production medium containing (g/L) 80.0 g sucrose, 0.9 g yeast extract, 6.0 g K_2HPO_4, 0.6 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$ and 4.0 g NaCl. 5 mM adenine, guanine, cytosine and uracil was separately added at 0 h, 24 h, 48 h and 72 h. 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. Temperature, pH, dissolved oxygen, agitation speed and aeration rate in the vessel could be monitored and controlled automatically. The fermentor with 3 L of the production medium was sterilized at 121 °C for 15 min. After cooling, the medium was inoculated with 150 mL of the seed culture. The pullulan production was carried out at constant temperature of 28 °C, aeration rate of 3 L/min and agitation speed of 300 rpm. The pH was controlled at 6.5 by feeding with either 2 M NaOH or 2 M HCl.

2.3. Isolation of exocellular polysaccharide

The fermentation broth was appropriately diluted with distilled water and heated at 80 °C in water bath for 30 min. The heated culture was centrifuged at 10,000 × *g* for 20 min to remove the microorganisms and other precipitates. The supernatant was mixed with two volumes of cold 95% (v/v) ethanol and held at 4 °C for 24 h to precipitate the pullulan completely. The precipitate was separated by centrifugation at 15,000 × *g* for 20 min, rewashed with ethanol, recovered by filtration and dried at 80 °C to a constant weight. The biomass pellet from the initial centrifugation was washed three times with distilled water by centrifugation at 10,000 × *g* for 5 min and dried at 80 °C until the dry cell weight (DCW) was constant. DCW and pullulan content were expressed as g/L.

2.4. Purification of pullulan

The dried exocellular polysaccharide samples were redissolved with distilled water (5%, w/v). The solution was dialysed using the dialysis tube of 10,000 molecular weight cutoff at 4 °C for 48 h to remove small molecules, with changing of distilled water thrice during the period. These samples were freeze-dried (Model: FreeZone6L, Labconco, USA) for analyses.

2.5. Analytical methods

After removing the pullulan by ethanol precipitation from the cell-free broth, the supernatant was used for the estimation of residual sucrose (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Ash, moisture, fat and protein content of the samples were determined as per standard methods (Anonymous, 1984).

2.6. Enzymes assays

UPase activity was assayed according to the method described by Ding et al. (2011). Briefly, an assay solution containing 2 mg wet cell paste, 10 mmol L^{-1} uridine, and 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) in a total volume of 2 mL was incubated at 60 °C for 20 min. The reaction was terminated by adding 1 mol L^{-1} ice-cold NaOH (2 mL). The reaction mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was diluted 50-fold with NaOH (pH 12). Enzymatic activity was determined spectrophotometrically by measuring the absorption at 290 nm relative to a blank. The blank solution was similar to the assay solution except that the cells were added after the addition of NaOH. Under the above reaction conditions, the specific activities of enzymes were expressed as a 0.01 change in absorption at 290 nm of the reaction mixture per gram wet cell paste.

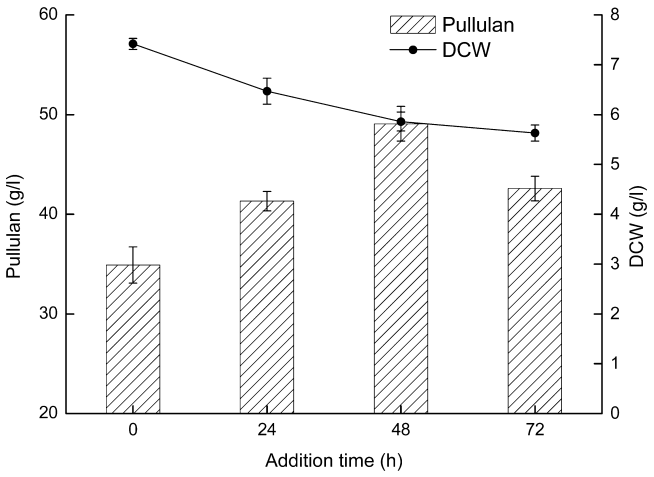

Fig. 1. Effect of addition time of uracil on pullulan production and cell growth by *Aureobasidium pullulans* CGMCC1234. Data are shown as mean ± SD (*n* = 3).

3. Results and discussion

3.1. Effect of nucleotide bases on pullulan fermentation

Supplementation of the adenine, guanine and cytosine had no encouragement on pullulan production, but increased the biomass production (date not shown). The addition time of uracil was important to increase pullulan production (Fig. 1). When uracil was added at 0 h, it was merely utilized as nitrogen nutrient source to stimulate cell growth, but reduced the pullulan production (blank control: 5.44 g/L DCW, 37.52 g/L pullulan). While at 48 h, pullulan production metabolism was promoted and the pullulan production increased to 49.07 g/L compared to 37.52 g/L with the control. Similar results that the uracil addition time was vital to stimulated the curdlan production in *Streptococcus* mutants was reported (Lee & Lee, 2001).

3.2. Effect of uracil on the activity of UPase

Table 1 shows the effect of uracil on UPase activity at different fermentation time. UPase activity could not be detected no matter uracil existed or not at 0 h, therefore uracil did not participate in the pyrimidine salvage pathway and might be degraded to serve as nitrogen nutrient source to stimulate cell growth. When uracil was added at 48 h, UPase activity increased from 138 U mg^{-1} to 362 U mg^{-1}. It was known that uracil was a precursor of UDP-glucose. Furthermore, UDP-glucose was a key factor in pullulan synthesis. Added uracil possibly provided an additional glucosyl donor. Pullulan was reported as a secondary metabolite and begun

Table 1
Effect of uracil on UPase activity. Upase activity was detected at 5 h after adding uracil.

Addition time (h)	UPase activity (U/mg wet cells)	
	5 mM uracil	Control
0	Not detected	Not detected
24	104 ± 6	49 ± 3
48	362 ± 23	138 ± 8
72	153 ± 7	85 ± 5

Values are given as means ± SD (*n* = 3).

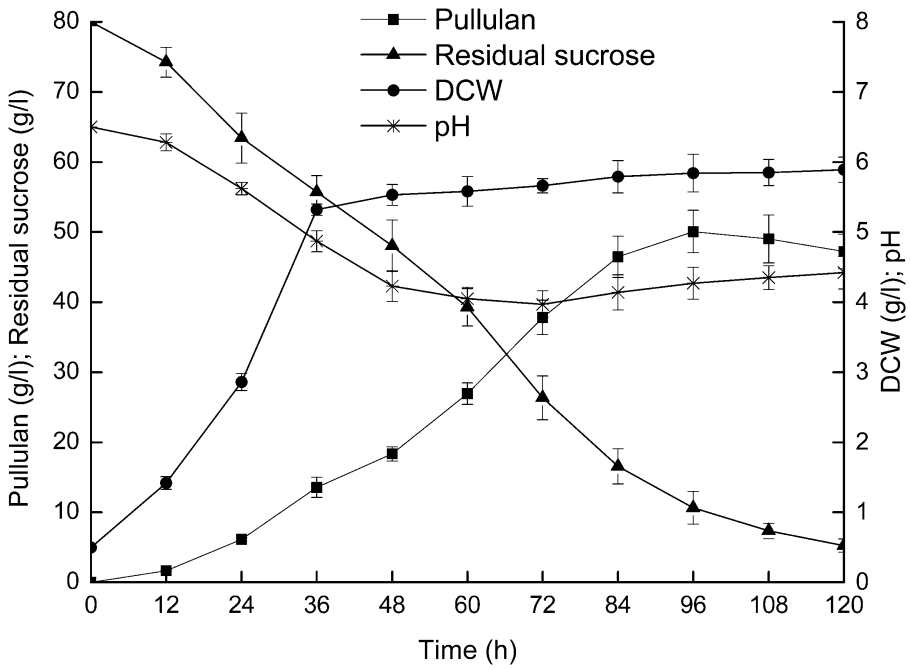

Fig. 2. Kinetics of pullulan fermentation of *Aureobasidium pullulans* CGMCC1234 in medium with adding 5 mM uracil at 48 h. Data are shown as mean ± SD ($n = 3$).

to be produced in late exponential phase; it could be produced more when growth became limited or as the cells approached a stationary phase (Pouliot, Walton, Nolen-Parkhouse, Abu-Lail, & Camesano, 2005). Only the physiological state of cell completely changed from growth to pullulan production, the added uracil could be used for improving the pullulan productivity.

3.3. Kinetics of pullulan production

The time course study on the production of pullulan by *A. pullulans* CGMCC1234 was made for a period of 120 h in the fermentation media prepared by adding 5 mM uracil at 48 h (Fig. 2). The biomass concentration sharply increased during the first 24 h of fermentation and continued to increase slightly till the end of the experimental period. There was a concomitant increase in the production of pullulan and decline in the residual sugar content in the fermentation medium. The maximum pullulan yield was observed at 96 h, and thereafter the pullulan yield slightly decreased. This phenomenon demonstrated that pullulan production was not closely associated with cell growth. The decrease of pullulan yield might be attributed to the activity of a pullulan-degrading enzyme at the late stage of pullulan fermentation (Zheng, Campbell, McDougall, & Seviour, 2008).

4. Conclusion

Uracil, acting as a precursor of UDP-glucose, was found to serve as an activator on the production of pullulan. The uracil addition time was crucial to increase pullulan production. Uracil enhanced the production of pullulan was associated with the UPase activity.

Acknowledgments

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