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Short communication

# Mechanism study of Tween 80 enhancing the pullulan production by *Aureobasidium pullulans*

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ABSTRACT

The influences of Tween 80 on the pullulan production, cell growth, glucosyltransferase activity and fatty acid composition of the cells were studied. The addition of Tween 80 to the culture medium significantly enhanced the pullulan production, but had no impact to biomass accumulation. Glucosyltransferase activity involved in pullulan synthesis was remarkable promoted with an increase of Tween 80 content. The ratio of unsaturated/saturated fatty acids contained in the cells was significantly higher in 0.5% Tween 80 in comparison with the absence of Tween 80. Results indicated that the increase of pullulan yield brought by Tween 80 was highly correlated with glucosyltransferase activity and fatty acid composition. Tween 80 was not degraded to serve as extra carbon source.



## 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 non toxicity, low viscosity, high plasticity and good film-forming. This polysaccharide is widely used in food and pharmaceutical industries (Cheng, Demirci, & Catchmark, 2011).

Pullulan production was under complex metabolic and environmental control. It was reported that pullulan chains originated from UDP-glucose (Shingel, 2004). Cately and McDowell (1982) had proposed the following order of the biochemical events preceding pullulan formation. The first stage was UDPG-mediated attachment of a D-glucose residue to the lipid molecule (LPh) with a phosphoester bridge. A further transfer of the D-glucose residue from UDP-glucose gave lipid-linked isomaltose. In the next step, isomaltose participated in the reaction with lipid-linked glucose to yield an isopanosyl residue. Further, isopanosyl residues were polymerized into the pullulan chain (Daran, Bell, & Francois, 1997). Therefore, glucosyltransferase might play an important role in biosynthesis of pullulan with high yield.

Tween 80 was a non-ionic surfactant and known as polyethylene glycol sorbitan monooleate. It had been reported that Tween 80 had the capability to enhance glucosyltransferase synthesis in *Streptococcus mutants* (Tomita et al., 1998) and *Leuconostoc dextranicum* (Majumder & Goyal, 2008). Tween 80 was also found to stimulate the production of pullulan in *A. pullulans*, although there was inadequate information available for explaining this phenomenon (West & Reed-Hamer, 1995). It had been confirmed that the activity of glucosyltransferase was highly correlated with the amount of pullulan produced and the carbon source used (Duan, Chi, Wang, & Wang, 2008). However, the relationships among Tween 80 concentration, glucosyltransferase activity and pullulan production have not been studied.

The objectives of this paper were to investigate the effect of Tween 80 on the pullulan production, biomass accumulation and glucosyltransferase activity of *A. pullulans* CGMCC1234. The effect of Tween 80 on the fatty acid composition of the cells was also evaluated.

## 2. Materials and methods

### 2.1. Organisms and inoculum preparation

*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

*Abbreviations:* *A. pullulans*, *Aureobasidium pullulans*; UDP-glucose, uridine diphosphate glucose; PDA, potato dextrose agar; DCW, dry cell weight.

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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 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 $MgSO_4$, 4.0 g NaCl and 0–10.0 g Tween 80. 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. Preparation of cell-free extract

The cells in 5.0 ml of the culture were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C. The cell pellet was washed three times with ice-cold distilled water by centrifugation at 10,000 × *g* for 5 min. The pellet was suspended in 1.0 ml of ice-cold 1.0 M Tris–HCl (pH 7.6) to make a thick paste. The paste was homegenized in a Homegenizer (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 (the cell-free extract) was stored at −80 °C and used as a source of enzymes. Protein concentration in the cell-free extract was determined by the method of Bradford with bovine serum albumin as standard (Bradford, 1976).

### 2.5. Enzymes assays

Glucosyltransferase activity was determined according to methods described by Duan et al. (2008). A suitably diluted enzyme preparation (0.2 ml) was incubated with pre-equilibrated *p*-nitrophenyl α-D-glucopyranoside (0.2 ml, 10 mM) in 0.1 M sodium acetate buffer (pH 4.0) for 5 min at 40 °C. The reaction was terminated by the addition of an aqueous solution of Trizma base (3.0 ml, 2.0%, w/v), and the absorbance at 410 nm was measured. Enzyme activity was expressed in terms of μM of *p*-nitrophenol released/min and calculated by reference to a standard curve (the standard curve was made by measuring $OD_{410\,nm}$ values of the solutions with different concentrations of *p*-nitrophenol). The mixture with the diluted enzyme preparation that had been heated at 100 °C for 5 min was used as the control.

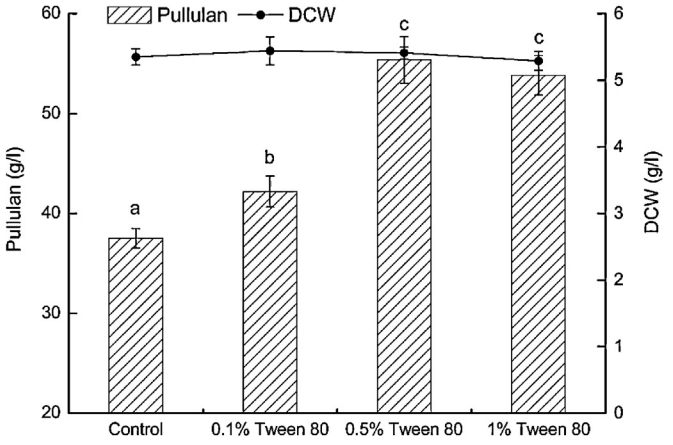


**Fig. 1.** Effect of Tween 80 concentration on pullulan production and cell growth by *Aureobasidium pullulans* CGMCC1234. Values are given as means ± standard deviation ($n = 3$). Bars denoted with different capital letters are significantly different ($P < 0.05$).

### 2.6. Lipid analysis

Cells harvested at the late-logarithmic phase were washed three times with the distilled water by centrifugation at 10,000 × *g* for 5 min. Total lipids extracted from the freeze-dried cells by the method of Blight and Dyer (1959) were methylated based on the report of Ahn et al. (2012). Fatty acids methyl esters were analyzed by gas chromatography as reported previously (Ahn et al., 2012).

## 3. Results and discussion

### 3.1. Effect of the concentration of Tween 80 on pullulan fermentation

Influence of various concentrations of Tween 80 on pullulan production and cell growth by *A. pullulans* CGMCC1234 are shown in Fig. 1. The addition of Tween 80 to the nutrient medium significantly increased the pullulan production. The pullulan production increased as the Tween 80 concentration in the medium increased. Certain experimental results evidenced that glucose-containing lipid intermediates played a crucial role in pullulan biosynthesis (Shingel, 2004). The lipid was used as a carrier in which pullulan was taken to the outside of the plasmalemma. The pullulan arranged in a network covering outer surface of the cells. Tween 80 was a well-known industrial surfactant, therefore it was assumed that its presence in the media would affect the homogeneity of the broth and facilitate the nutrient and oxygen transfer to the microorganism. However, the addition of Tween 80 to the nutrient medium had no effect on biomass production, indicating that Tween 80 was not degraded to serve as extra carbon source.

### 3.2. Effect of the concentration of Tween 80 on glucosyltransferase production

Effect of different concentrations of Tween 80 on glucosyltransferase activity is shown in Fig. 2. Glucosyltransferase activity was remarkably promoted with an increase of Tween 80 content. Furthermore, when Tween 80 was added to the cell-free extract, no

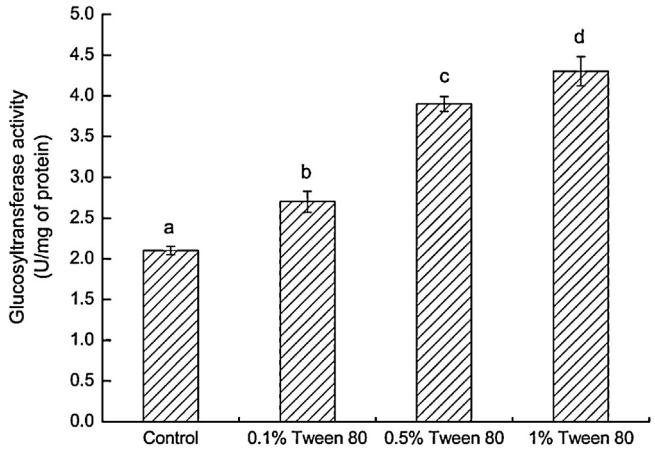


**Fig. 2.** Effect of Tween 80 concentration on glucosyltrasferase activity. Values are given as means ± standard deviation ($n = 3$). Bars denoted with different capital letters are significantly different ($P < 0.05$).

**Table 1**
Fatty acid composition of *A. pullulans* cells grown with and without Tween 80.

| Fatty acids | % Total fatty acids | |
|---|---|---|
| | Control | 0.5% Tween 80 |
| C16:0 | 23.7 ± 1.2 | 16.0 ± 0.4* |
| C16:1 | 1.3 ± 0.1 | 4.3 ± 0.2* |
| C18:0 | 6.2 ± 0.3 | 4.4 ± 0.1* |
| C18:1 | 34.8 ± 0.9 | 55.7 ± 1.0* |
| C18:2 | 34.0 ± 0.7 | 19.6 ± 0.5* |
| Unsaturated/saturated[a] | 2.34 | 3.90 |

Values are given as means ± standard deviation ($n = 3$).
* Significant difference between control and treatment group ($P < 0.05$).
[a] Ratio of unsaturated/saturated = (C16:1 + C18:1 + C18:2)/(C16:0 + C18:0).

increase in the glucosyltransferase activity was observed (date not shown). As a result of this, Tween 80 did not affect the activity of glucosyltransferase by converting the enzyme from an inactive form to an active form and thus the increased glucosyltransferase activity might be due to the improvement of glucosyltransferase production in presence of Tween 80. Similar results that Tween 80 stimulated the production of glucosyltransferase and had no direct activating effect on enzyme activity in *S. mutants* was reported (Umesaki, Kawai, & Mutai, 1977).

### 3.3. Effect of Tween 80 on fatty acid compositions

Table 1 shows the effect of Tween 80 on the fatty acid composition of the cells grown to the late-logarithmic phase. Enhancement of extracellular pullulan production and intracellular glucosyltransferase activity by Tween 80 was associated with changes in fatty acid composition in the cells. The major fatty acids contained in the cells were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). In the presence of Tween 80, the ratio of unsaturated/saturated fatty acids increased from 2.34 to 3.90. The increase in the unsaturated degrees by Tween 80 was mainly due to the result that oleic acid increased with a decrease of palmitic acid. The interaction of Tween 80 with cells affected the permeability of the cell membrane, which might enhance to uptake the essential components including vitamins from surroundings (Taoka et al., 2011) and to release of pullulan into the extracellular fluid.

## 4. Conclusions

The addition of Tween 80 to the culture medium enhanced pullulan production, but had no effect on biomass production. The mechanism by which Tween 80 enhanced the production of pullulan was associated with the glucosyltransferase activity and fatty acid composition.

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