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Influence of Tween-80 on the production and structure of water-insoluble curdlan from *Agrobacterium* sp.

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ABSTRACT

In order to explore the mechanism by which Tween-80 enhances the production of curdlan produced by *Agrobacterium* sp., the effects of Tween-80 on the production and structure of curdlan and *Agrobacterium* sp. were evaluated. Maximum curdlan production (51.94 g/L) was achieved when 16 g/L Tween-80 was added at the beginning of the cell growth stage. The addition of Tween-80 at higher concentration inhibited cell growth. However, the addition of 16 g/L Tween-80 enhanced the production of curdlan with a looser ultrastructure, significantly weakened the envelopment of curdlan on *Agrobacterium* sp., altered the fine structure of cell membrane, and increased the cell membrane permeability. Moreover, the efficiency of oxygen and mass transport, respiration intensity, UTP regeneration, ATP regeneration, activity of curdlan synthetase, capacity of stress response and energy supply of *Agrobacterium* sp. were all greatly improved by the addition of Tween-80. These findings demonstrate the mechanisms by which Tween-80 enhances curdlan production and provide a cheap and feasible approach to weaken the envelopment of water-insoluble polysaccharides on bacteria.

1. Introduction

Curdlan is a water-insoluble extracellular polysaccharide composed of β-1, 3-linked glucose residues with no branching [1,2]. Aqueous suspensions of curdlan can be thermally induced to produce gels that do not return to the liquid state, demonstrating unique rheological and thermal gelling properties. Because of these characteristics, curdlan has great commercial applicability in the food and pharmaceutical industries [2,3].

Curdlan is commercially produced by *Agrobacterium* sp. under non-growing, nitrogen-limiting conditions [4]. To meet the increasing demand for curdlan, it is necessary to improve the efficiency of curdlan production [5]. Numerous approaches have been developed to improve curdlan production, including screening for high-yield mutant strains [6], optimization of culture medium [7–10], and control of pH and oxygen supply [11]. Despite these efforts, the serious envelopment of curdlan on *Agrobacterium* sp. remains a major obstacle to enhancing cell metabolism and increasing curdlan production [11]. In our previous study [12], a coupled fermentation system of *Agrobacterium* sp. and *Trichoderma harzianum* GIM 3.442 was successfully established to weaken the envelopment of curdlan on *Agrobacterium* sp. and produce low-molecular-weight curdlan at high yields. We have also focused on developing methods to weaken the envelopment of curdlan on *Agrobacterium* sp. and enhance curdlan production using a single fermentation system of *Agrobacterium* sp.

Surfactants are amphiphilic compounds that can decrease surface and interfacial tensions by accumulating at the interface of immiscible fluids [13,14]. Surfactants have been reported to enhance the production of β-glucan-type exopolysaccharides, such as pullulan [15], gellan gum [16], xanthan [17], and welan gum [18]. Some of the proposed mechanisms through which surfactants increase polysaccharide production are by increasing cell membrane permeability, increasing glucosyltransferase activity, altering lipid metabolism and dissolving lipid molecules to enable the release of the polysaccharide [16,19].

Tween-80 (polyethylene glycol sorbitan monooleate) is one of the most important non-ionic surfactants. It has been reported that Tween-80 is capable of enhancing the production of several bioconversion products [20–22]. To our knowledge, there has been only one previous report evaluating the effect of Tween-80 on curdlan production by *Alcaligenes faecalis* ATCC 31749 [23]. Xia reported

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that the addition of Tween-80 at a low concentration promoted curdlan production but had no effect on cell growth. The enhanced curdlan production by Tween-80 was highly correlated with glucosyltransferase activity. However, there is little information about the effect of Tween-80 on the structure of curdlan, as well as certain aspects of *Agrobacterium* sp., including morphology of cell membrane and cell metabolism.

Therefore, in this study, we attempted to weaken the envelopment of curdlan on *Agrobacterium* sp. and enhance curdlan production using Tween-80 in a single fermentation system of *Agrobacterium* sp. The concentration and time of addition of Tween-80 were optimized, and then the structures of curdlans produced with or without Tween-80 addition were compared by small-angle X-ray scattering (SAXS), Fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR). Moreover, the effect of Tween-80 on the cell membrane, intracellular nucleotide levels, and transcription levels of several genes were investigated to further explore the mechanisms by which Tween-80 enhances curdlan production. This study presents information on the mechanisms by which Tween-80 enhances curdlan production and additionally provides a cheap and feasible approach to weaken the envelopment of water-insoluble polysaccharides on bacteria in a single fermentation system.

2. Materials and methods

2.1. Microorganism

Agrobacterium sp. ATCC 31749 was obtained from the Key Laboratory of Carbohydrate Chemistry and Biotechnology of Jiangnan University.

2.2. Medium

2.2.1. Shake-flask culture

The seed medium contained 20 g/L glucose, 10 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 1.74 g/L KH_2PO_4, and 0.5 g/L $MgSO_4$ at a final pH of 7.0. The fermentation medium contained 50 g/L glucose, 3 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2.7 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, and 10 g/L $CaCO_3$ at a final pH of 7.0.

2.2.2. 7 L-fermenter culture

The seed medium was the same as that used for the shake-flask culture. The fermentation medium contained 100 g/L glucose, 6 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2.7 g/L KH_2PO_4, and 0.5 g/L $MgSO_4$ at a final pH of 7.0.

2.3. Culture methods

Agrobacterium sp. was incubated on an agar plate at 30 °C for 48 h. Following this, a single colony was inoculated in 50 mL of seed medium and cultured at 30 °C and 200 rpm for 16 h. After this, for the shake-flask culture, 5 mL of the seed culture solution was inoculated into 50 mL of fermentation medium and incubated at 30 °C and 200 rpm for 72 h. For the 7 L-fermenter culture, the seed culture solution was inoculated into the fermentation medium (10%, v/v) in a 7 L fermenter (BioFlo 115, New Brunswick, England) for fed-batch cultivation at 30 °C. The fed-batch cultivation consisted of cell growth stage and curdlan production stage. During the cell growth stage, the stirring rate, airflow rate, and pH were set at 400 rpm, 2 vvm, and 7.0, respectively. The ammonia nitrogen was depleted when the dissolved oxygen (DO) level suddenly decreased. Nitrogen depletion is the signal for the initiation of curdlan synthesis. During the curdlan production stage, the pH was set at 5.6 and the agitation speed was set varying from 400 to 800 rpm in order to keep the DO level more than 50%. The DO level, pH, temperature, and impeller speed were recorded using Advanced Fermentation Software from New Brunswick Scientific Co. Inc.

2.4. Determination of residual ammonia nitrogen level

The residual ammonia nitrogen level was determined using the indophenol blue method [24]. Solution A (5 mL) (10 g/L phenol and 0.05 g/L sodium nitroprusside) was mixed with 10 μL of the sample. After adding 5 mL of solution B (5 g/L NaOH, 7 mL/L NaClO, and 4 g/L trisodium citrate), the mixture was incubated at 37 °C for 20 min and the absorbance was measured at 637 nm. The standard curve was $y = 5.494x - 0.0334$ ($R^2 = 0.9997$), where y is the concentration of $(NH_4)_2SO_4$ solution, and x is OD_{637}.

2.5. Determination of biomass and curdlan production

Biomass and curdlan production were determined by the dry weight method [25]. 15 mL of fermentation broth was centrifuged at $6010 \times g$ for 20 min, and the pellet containing curdlan and *Agrobacterium* sp. was resuspended in 20 mL of 1 mol/L NaOH with stirring at 200 rpm for 3 h. Then the cells were precipitated by centrifugation at $6010 \times g$ for 20 min and curdlan was dissolved in the supernatant. The pH of the supernatant was adjusted to 7.0 using 2 mol/L HCl, and curdlan was then precipitated after centrifugation at $6010 \times g$ for 20 min. The cells and curdlan were both repeatedly washed with deionized water and dried to a constant weight at 105 °C.

2.6. Determination of glucose concentration

The glucose concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method [26]. The procedure was as follows: 0.5 mL of sample and 1.5 mL of DNS solution (6.5 g/L 3,5-dinitrosalicylic acid, 26 g/L NaOH and 45 g/L glycerol) were mixed and boiled at 100 °C for 10 min. The mixture was then diluted to 25 mL and the absorbance was measured at 540 nm. The standard curve was $y = 0.5588x - 0.0045$ ($R^2 = 0.9995$), where y is OD_{540} and x is the glucose concentration. The glucose concentration was calculated according to the standard curve.

2.7. Structural characterization of curdlan

The monosaccharide composition was determined using the method described in Liu et al. [27]. The fiber period of the single helical structure of curdlan was measured by SAXS (NanoSTAR, Bruker, Germany). The specimen was prepared using the method described in Takeda [28]. The fiber period was calculated using Eq. (1)

$$d = 2\pi/q \quad (1)$$

where d and q are the fiber period and scattering vector, respectively.

FT-IR was performed using a Fourier transform infrared spectrometer (NEXUS, Thermo., US) with a single-reflection diamond attenuated total reflection and a mercury-cadmium-telluride detector was used. The spectrum was recorded from 378 to 4000 cm^{-1} at 32 scans and 0.5 cm^{-1} resolution [29]. The curdlan was dissolved in deuterated dimethyl sulphoxide and analyzed using an NMR spectrometer (Avance III, Bruker, Germany) at 400 MHz [27].

2.8. Transmission electron microscopy (TEM) observation of curdlan

The morphology of the curdlan layer was observed by TEM (H-7650, Hitachi, Japan) at an accelerating voltage of 80 kV. One drop

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