Accumulation of Cello-oligosaccharides during Bacterial Cellulose Production by *Acetobacter xylinum*

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Abstract: *Acetobacter xylinum* ATCC23769 produces not only cellulose but also various oligosaccharides during cell growth. These oligosaccharides accumulated and increased gradually up to about half the amount of cellulose with the increase of endo-1,4-α-glucanases activity in culture broth. These oligosaccharides were identified cello-oligosaccharides, gentiobiose and rhamnose, which were constituent sugars of acetan. It is suggested that they are the degradation products from acetan, as enzymes prepared from culture broth hydrolyzed acetan rather than cellulose.

Key words: *Acetobacter xylinum*, cello-oligosaccharides, cellulose, acetan

*Acetobacter xylinum* has been used for many years as a model system for the study of cellulose biosynthesis. Interestingly, in addition to cellulose biosynthesis, this bacterium has also produced cellulolytic enzymes.11 However, the relation between the two products has not been made clear. Furthermore, it is reported that some strains of *Acetobacter* have accumulated a water-soluble polysaccharide called acetan,2 which has a similar structure to xanthan.3

Cellulose is an insoluble β-1,4-glucan, but acetan is a soluble sugar, which has the same backbone as cellulose. Furthermore it has attached side chain composed of glucose, mannose, glucuronic acid and rhamnose and the composition of those sugars is 4:1:1:1 (glucose:mannose:glucuronic acid : rhamnose).

It was reported that *Acetobacter acetigenum* produced various carbohydrates, cellubiose, cellotriose, cellotetraose and fructose growing in a defined medium containing glucose.5 Valla et al.6 have shown a new extracellular polysaccharide from a cellulose-negative strain of *A. xylinum* ATCC10245. In that report they showed that the polysaccharide contains glucose, mannose, rhamnose, and glucuronic acid in a molar ratio approximating 3:1:1:1. Partial acid hydrolysis results in release of glucose, rhamnose, and a disaccharide consisting of two glucose units. They speculated that this disaccharide might be gentiobiose, but the origin of these sugars has not been clarified. We also detected gentiobiose and various cello-oligosaccharides in the culture broth of *A. xylinum*. In this paper we discuss what has been the real origin of various cello-oligosaccharides synthesized during culture.

First, the sugars produced in the Hestrin and Schramm (SH) medium during the static culture of *A. xylinum* ATCC23769 have been analyzed. Changes in the amounts of cello-oligosaccharides, acetan, cellulose, endo-1,4-α-glucanase activity in culture solution and cell growth during the culture are shown in Fig. 1. In the static culture, cell mass did not increase linearly but showed as the shape of stairs. The products such as cellulose, cello-oligosaccharide and endo-1,4-α-glucanase also increased in the same pattern. It seems that cell division and the matter production occurred in turn, and consequently cellulose produced was composed of the thick of individual sheet. Various cello-oligosaccharides and endo-1,4-α-glucanase were produced after one day’s culture and increased gradually. Cellulose production showed the same tendency as cello-oligosaccharides and endo-1,4-α-glucanase productions, and was related closely. During the culture *A. xylinum* produced 0.2 mg of cello-oligosaccharides as the total amount after one day and finally over 1.5 mg/mL of them at 2 weeks. It is interesting that cellulose, cellulose degrading enzyme and cello-oligosaccharides, which might be produced from cellulose by enzyme degradation, were closely related to each other.

Cello-oligosaccharides were detected in culture broth of *A. xylinum* as shown in Fig. 2. A small amount of cellulotriose, cellotetraose and cellopentaose were detected in the fraction eluted by 15, 20 and 25% of ethanol at the charcoal column chromatography, respectively. In addition to cello-oligosaccharides, rhamnose was detected. Moreover, a spot just like celllobiose was detected by several TLC analyses (data not shown).

β-Glucodisaccharides were identified by ABEE (p-aminobenzonic acid ester)-conversion method7 because a spot just like celllobiose tailed and was detected strongly (Fig. 2). The chromatogram showed that β-glucodisaccharides as β-1,4-cellobiose and β-1,6-gentiobiose were surely produced by *A. xylinum* (Fig. 3).

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This strain is known not to accumulate a water-soluble polysaccharide called acetan. To investigate the origins of these cello-oligosaccharides, we tried to degrade acetan by using enzymes produced in the culture, which were prepared by the method of Ishida et al. We have investigated the properties of a partially purified enzyme, \( \beta \)-glucanase, produced by \textit{A. xylinum}. Time courses of reducing sugar formation by the partially purified enzyme on acetan, carboxymethyl-cellulose (CMC) and bacterial cellulose (BC) are shown in Fig. 4. The \( \beta \)-glucanases

\section*{Fig. 1. Changes in cellulose production, the amounts of cello-oligosaccharide formation, acetan formation, cell mass and endo-1,4-\( \beta \)-glucanase activity of \textit{A. xylinum} ATCC23769.}

The cell mass was estimated by measuring the optical density at 660 nm after cellulose dissolved by treating it with endo-1,4-\( \beta \)-glucanase (Celluclast, Novo).

Cellulose was treated with 2% NaOH to dissolve cells and washed with distilled water, after which the amount of cellulose produced was measured and then the dry weight of the cellulose sheet was measured. The amounts of cello-oligosaccharides in the culture broth were determined using HPLC.

The culture solutions were applied on charcoal column, washed with distilled water and then eluted with 5, 10, 15, 20, and 25 wt% ethanol to fractionate a small amount of cello-oligosaccharides. Thirty \( \mu \text{L} \) aliquots of culture broth were spotted on analytical thin layer chromatography (TLC) plates (Silicagel 60, 0.25 mm, Merck). TLC was performed by using the double-ascending method with a solvent system of chloroform–methanol–water (90 : 65 : 15). The TLC plate was sprayed completely with 30 wt% of H\(_2\)SO\(_4\) and then heated at 130°C for 5–10 min to detect cello-oligosaccharides. Symbols: S, standard sugars; G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; R, rhamnose; numbers, fractions eluted on charcoal column chromatography; 5, 5% EtOH; 10, 10% EtOH; 15, 15% EtOH; 20, 20% EtOH; 25, 25% EtOH.

\section*{Fig. 2. Thin layer chromatogram of oligosaccharides produced by \textit{A. xylinum} ATCC23769.}

\section*{Fig. 3. HPLC profiles of \( \beta \)-glucodisaccharides produced by \textit{A. xylinum} ATCC23769.}

\( \beta \)-Glucodisaccharide was modified to an aminobenzoic acid ethyl ester of \( \beta \)-glucodisaccharide by reductive amination. An ABEE reagent solution (40 \( \mu \text{L} \)) was added to the culture (10 \( \mu \text{L} \), and mixed by vortex and then cooled to room temperature. Equal volumes of chloroform were added before adding distilled water (0.2 mL). After vigorous mixing, the mixtures were centrifuged for 3 min, and the upper aqueous layer was subjected to HPLC analysis. ABEE-converted oligosaccharide analysis was done using a UV monitor (SPD-6A, Shimadzu, Kyoto) at the wavelength of 305 nm with an Honenpak C18 column (4.6×75 mm, Seikagaku, Tokyo). The mobile phase was 0.1 M ammonium acetate buffer (pH 4.0) contained acetonitril at the flow rate of 1.0 mL/min. Peaks of 1 and 2: ABEE-converted \( \beta \)-1,6-gentiobiose and \( \beta \)-1,4-cellobiose, respectively.
could attack acetan and CMC, but not BC. The amounts of reducing sugars from acetan were larger than those from CMC and BC. From this result it is suggested that cello-oligosaccharides accumulated could not be hydrolysis products from BC.

What is the real original source of cello-oligosaccharides? It is proposed that these sugars are the degradation products not from cellulose but from acetan, because this strain secreted extracellular enzyme for acetan degradation. We examined degradation of acetan by the partially purified enzyme, \( \beta \)-glucanase. These enzymes degraded acetan, and therefore various oligosaccharides which are constituent sugars of acetan were detected as hydrolysis products. However, we can’t deny another possibility that they are intermediate products for acetan biosynthesis. This strain might lack the ability for polymerization of repeating units of acetan, though this strain could synthesize the oligosaccharides which are the minimum unit for acetan synthesis. Recently a few genes encoding enzymes to synthesize acetan have been well characterized. \( ^{10-13} \) Ishida et al. \( ^{14} \) isolated aceQ and aceR genes involved in acetan biosynthesis. They also obtained a aceQ-disrupted mutant and aceR-disrupted mutant. Both mutants secreted acetan analogs lacking various side chains. It is supposed that \( A. \ xylinum \) ATCC23769 may lack these genes and therefore oligosaccharides which are constituents of acetan were detected instead of a water-soluble polysaccharide accumulation. In the near future we expect to have clear results about these sugar’s formations.

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