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Article

The relationship between cellulase activity and oligosaccharides and cellulose productions by *Acetobacter xylinum* ATCC23769

Fuyu Ito, Yoshihiko Amano*, Kouichi Nozaki, Inder M. Saxena[1],
Malcolm R. Brown Jr.[1], Takahisa Kanda

Department of Chemistry and Material Engineering, Faculty of Engineering, Shinshu University (4-17-1, Wakasato, Nagano 380-8553, Japan)
[1]*School of Biological Science, the University of Texas at Austin (Austin, Texas 78712, USA)*

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We have tried to investigate various carbohydrates production and cellulose degrading enzyme activity in the culture broth of cellulose producing microorganism, *Acetobacter xylinum* to clarify the role of cellulase for cellulose production. Carboxymethylcellulose (CMC) degrading activity and various oligosaccharides in addition to cellulose have already been detected after one-day culture. These oligosaccharides increased gradually during the culture, were identified to be rhamnose, mannose, some kinds of β-linked disaccharides such as β-1, 4-linked and β-1, 6-linked glucobiose (cellobiose and gentiobiose), cellotriose and cellotetraose by thin layer chromatogram analysis. It is assumed that these oligosaccharides may be enzymatic degradation products from acetan produced in the culture broth because they are the constitution sugar of acetan and cellulase produced by this microorganism could not degrade bacterial cellulose produced by itself. Cellulase activity is correlated closely with cellulose production, and more closely related to the changes in amount of oligosaccharides. In addition when *A. xylinum* was cultured in the presence of β-glucodisaccharides such as gentiobiose and cellobiose, we observed the increases of cellulase activity and cellulose production.

Keywords: *Acetobacter xylinum*, cellulase, β-oligosaccharides

*Corresponding author
(Tel/Fax: +81-26-269-5394, E-mail: yoamano@gipwc.shinshu-u.ac.jp)

Introduction

Cellulose, the most abundant organic polymer on the earth, occurs in plants and certain algae, fungi, and bacteria.[1] It is an extracellular polysaccharide, synthesized as long β-1,4 glucan chains that associate to form the microfibrils commonly observed in cellulose-synthesizing organisms. It is well known that some strains of *Acetobacter* sp. produce bacterial cellulose (BC).[2, 3] The gram-negative bacterium *Acetobacter xylinum* synthesizes an extracellular ribbon of cellulose microfibrils from membrane-localized cellulose synthesizing protein complexes that are organized in a linear row along the long axis of the bacterial cell. This BC is expected to be a new industrial material because of its unique characteristics such as ultra fine fibrils and the extremely high Young's modulus of a sheet prepared from it.[4] It is thought that further investigations on cellulose producing mechanism are essential to dissolve the problem of cost performance for industrial use.

Various reports have described the production of cellulolytic enzymes by cellulose-producing *Acetobacter* strains.[5] The role of this enzyme, however, has not been known clearly. Furthermore it is reported that *A. xylinum* has also produced various sugars and water-soluble polysaccharide called acetan in addition to cellulose.[6] The structure of acetan have been investigated, which is similar to xanthan produced by microorganism.[7, 8] Valla *et al*.[9] detected mannose, rhamnose, glucuronic acid, glucuronyl mannnose and disaccharide consisting of two glucose monomers in culture broth of *A. xylinum* in 1981. They thought this disaccharide was probably gentiobiose, but the origin of these sugars has not been clarified. In addition, it has been reported that some polysaccharides such as CMC could be altered the structure of cellulose produced.[10, 11]

In this paper, the metabolic products such as sugars and cellulase in the culture medium during culture of *A. xylinum* ATCC23769 have been investigated. In particular we investigated the relationship between cellulase activity and oligosaccharides produced in culture broth to clarify the role of cellulase for cellulose production.

Materials and Methods

Organism and media. *A. xylinum* ATCC 23769 was used in this study. For seed culture, Hestrin & Schramm (SH)[12] medium was used, in which SH was composed of 2% (w/v) glucose (Waco Chemicals, Tokyo), 0.5% (w/v) yeast extract (Difco Laboratories, USA), 0.5% (w/v) peptone (Nihon seiyaku, Tokyo), 0.27% (w/v) Na_2HPO_4 and 0.115% (w/v) citric acid (Waco). We have also prepared SH media containing 0.05 wt% of β-glucodisaccharides such as cellobiose and gentiobiose.

Cultivation. This study was performed using 5-d-old agar "rough" colonies[13] of strain ATCC23769. To examine metabolic products it was cultured in SH medium with of β-glucodisaccharides such as cellobiose and gentiobiose. After 40 ml of liquid medium in 100 ml Erlenmeyer flask was sterilized at 121℃ for 15 min, single colony was inoculated to each flask. It was incubated

at 25℃ for 14 days, and 400 μl of culture broth was collected and then used for analysis. After 100 ml of liquid medium with β-glucodisaccharide in 300 ml Erlenmeyer flask was sterilized at 121℃ for 15 min, and a colony was inoculated to investigate cellulase activity and cellulose production. The culture condition was the same as mentioned above.

Analytical methods.[13] The cell mass was estimated by measuring the optical density at 660 nm after dissolving cellulose by enzymatic treatment with cellulase (Celluclast, Novo). Cellulose produced by *A. xylinum* was treated with 2% NaOH at 100℃ for 30 min to dissolve cells and was washed with distilled water, to measure the amount of cellulose produced. The dry weight of cellulose sheet was measured after drying with filter papers at 50℃ for 24 h in vacuum oven. The amounts of glucose, gluconic acid and oligosaccharide in the culture broth were determined using HPLC. They were detected using a RI monitor (410; Waters, USA) equipped with a TSKgel G-Oligo-PW column (7.8×300 mm, TOSOH, Tokyo). Distilled water was used as a mobile phase at the flow rate of 0.5 ml/min (801, JASCO, Tokyo).

Assay of CMCase activity. The activity was assayed viscometrically in Ostwald-type viscometer at 30℃ with 1ml of the culture, 4 ml of 10 mM sodium acetate buffer (pH 5.0) and 1 ml of 1 wt% CMC (carboxymethylcellulose). One unit of cellulase activity was defined as amount of enzyme causing a 0.1 decrease in relative viscosity after 24 h incubation.

Thin-layer chromatography. The culture solutions were applied on charcoal column (3.0×75 cm), and washed with distilled water and then eluted with 5, 10, 15, 20, 25, 50% ethanol to detect a small amount of oligosaccharides. Thin-layer chromatography (TLC) was performed by using the double-ascending method with a solvent system of chloroform – methanol – water (90:65:15). TLC plates were sprayed completely with 30 wt% of H_2SO_4 solution and then heated at 130℃ for 5-10 min.

ABEE-conversion method. β-gluco-disaccharides were converted to corresponding aminobenzoic acid ethyl ester as (ABEE)-converted β-glucodisaccharide by reductive amination. An ABEE reagent solution (40 μl) was added to the culture solution (10 μl), and mixed with 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 (4.6×75 mm, Seikagaku, Tokyo). The mobile phase was 0.1 M ammonium acetate buffer (pH 4.0) containing 0.1 M acetonitril at the flow rate of 1.0 ml/min.

Hydrolysis of oligosaccharides The reaction mixture consisted of 30 μl of the fractions eluted with 5-15% ethanol, 30 μl of 5.0 Unit/mg β-glucosidase from almonds (SIGMA, USA). After incubation at 37℃ for 2 h, 30 μl aliquots of reaction mixtures were spotted on analytical TLC plates.

Results and Discussion

Cell growth and metabolic products in SH medium

Changes in the amounts of glucose, gluconic acid, cell mass and pH during the culture are shown in Fig. 1. The amounts of glucose in the culture broth were measured using HPLC analysis. In the initial stage, glucose as the sole carbon source appeared tube converted to gluconic acid. At 3rd day, pH of the culture gradually began to decrease from 6.0 to 4.0, where cell growth became active. After 5 days the pH of culture solution stayed constant around 4.0 and cells began to grow rapidly. Cell mass was reached at its maximal at 1.4 mg/ml after 2 weeks incubation, and the pH of the culture broth increased gradually from the lowest value of 3.8 to 5.0.

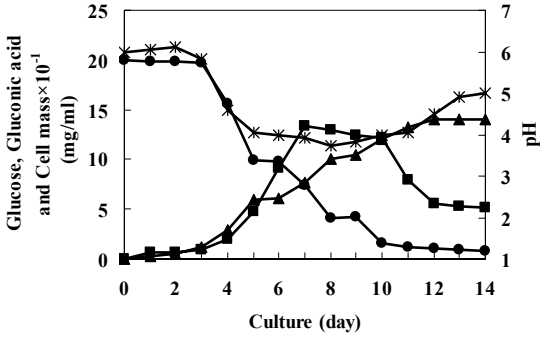

Fig. 1. Changes in the amounts of glucose, gluconic acid, cell mass and pH during the culture of *A. xylinum* ATCC23769.
Symbols: ●, glucose; ■, gluconic acid; ▲, cell mass; ✻, pH.

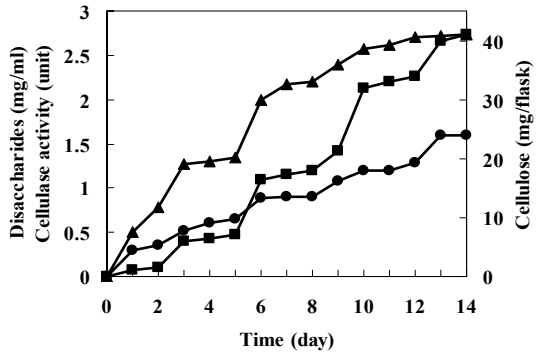

Fig. 2. Changes in cellulase activity, cellulose production and the amounts of oligosaccharides during the culture of A. xylinum ATCC23769.
Symbols: ●, disaccharides; ■, cellulose; ▲, cellulase activity.

Changes in cellulase activity, cellulose production and the amounts of oligosaccharides during the culture are shown in Fig. 2. 0.2 mg/ml of disaccharides were produced after 1 day and increased gradually thereafter. As compared with 5 and 6 days culture, the accumulation of disaccharides increased rapidly and the same phenomenon were observed between 8 and 10 days culture. Cellulose production showed a linear correlation with cell growth. At 14 days, the amount of cellulose was maximal at 40 mg/flask. On the other hand, the level of the extracellular cellulase activity increased rapidly after 1 day and was kept constant of the activity between 3 and 5 days culture. Then it increased rapidly again and this tendency was repeated. From these results it was found that the increases of cellulase activity, cellulose production and the amount of oligosaccharides are inclined to show the

similar changes during the culture. It has been assumed that oligosaccharides produced in the culture broth are degradation products of cellulose. However in the early phase of culture in this experiment, about 0.2 mg/ml of oligosaccharides were produced after 1 day incubation even though CMCase activity was very low and little cellulose has produced. So we have carried out in detecting a small amount of oligosaccharides in the culture as describing in the proceeding section.

Identification of β-glucodisaccharide in SH medium

During the culture this bacterium produced all sorts of sugars. Thin layer chromatogram of oligosaccharides produced is shown in Fig. 3. Rhamnose, mannose, disaccharide, cellotriose and cellotetraose were detected in the culture medium. Furthermore, HPLC profiles by ABEE-conversion of β-glucodisaccharide produced are shown in Fig. 4. These chromatograms showed the detection of disaccharides such as gentiobiose, cellobiose and a very little laminaribiose. Moreover hydrolysis products from these oligosaccharides by β-glucosidase were examined. These oligosaccharides were degraded to glucose (data not shown). Consequently these oligosaccharides were identified β-linked oligosaccharides.

It is not obvious what is real origin of those oligosaccharides. Various reports have described *A. xylinum* has also accumulated a water-soluble polysaccharide called acetan, the structure of which is similar to that of xantan.[3~5] Acetan consists of glucose, mannose, glucuronic acid, and rhamnose in the proportions of 4:1:1:1. However we confirmed that the strain we used, *A. xylinum* ATCC23769, did not produce acetan. It seems that this strain have no ability to polymerize the minimum unit of acetan. Therefore these various oligosaccharides may be secreted as metabolic products in the culture without polymerization. In future we hope to describe what the role of these oligosaccharides during cellulose production is.

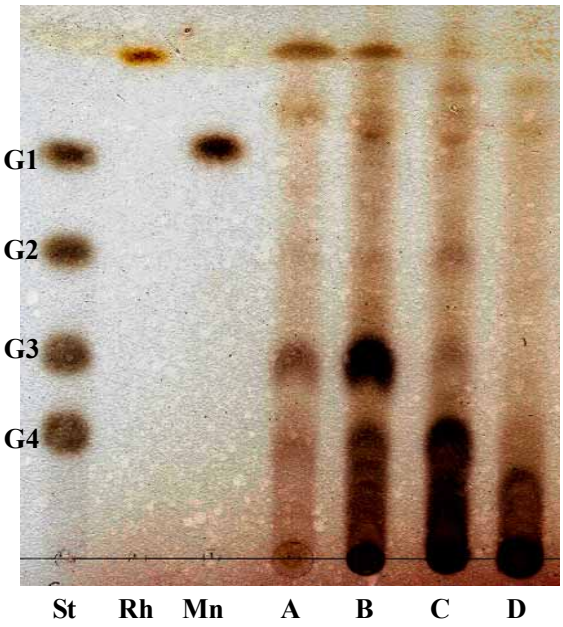

Fig. 3 Thin layer chromatogram of oligosaccharides produced by *A. xylinum* ATCC23769.
Symbols: S1: standard sugars, G1: glucose, G2:cellobiose, G3: cellotriose, G4: cellotetraose, Rh: rhamnose, Mn: mannose, A ～D: fractions eluted on charcoal column chromatography with various solvent as follows, A: 5% EtOH, B: 10% EtOH, C: 15% EtOH, D: 20% EtOH.

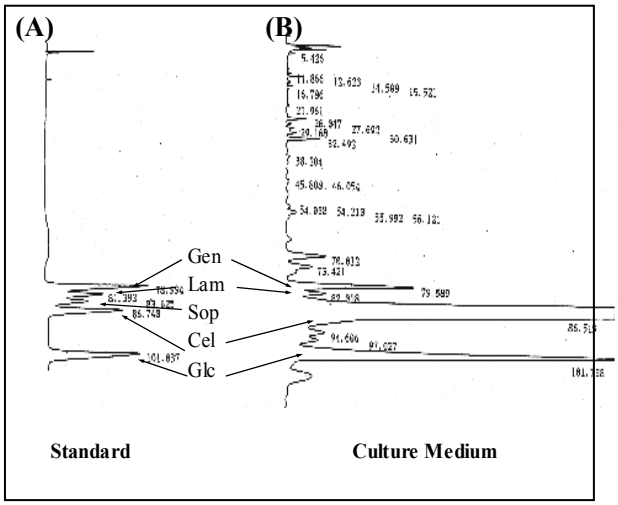

Fig. 4. HPLC profiles of β-glucodisaccharides produced by *A. xylinum* ATCC23769.
(A) profile of authentic sugars. (B) profile of culture broth for 7 days incubation.
Symbols: Gen, gentiobiose; Lam, laminaribiose; Sop, sophorose; Cel, cellobise; Glc, glucose.

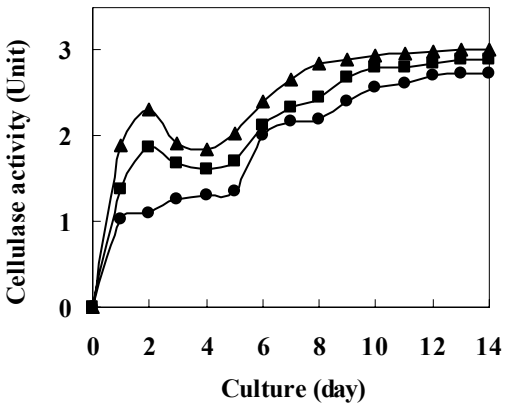

Fig. 5. CMC-degrading activity in three kinds of culture broths of *A. xylinum* ATCC23769.
Symbols: ●, SH medium; ■, SH medium with cellobiose; ▲, SH medium with gentiobiose.

Cell growth and cellulose production cultured in the presence of β-glucodisaccharides

Moreover the metabolic products cultured in the presence of β-glucodisaccharides such as cellobiose and gentiobiose have been investigated. Changes in cellulase activity during the culture are shown in Fig. 5. Cellulase activity produced in SH medium with cellobiose or gentiobiose was higher than that produced in SH medium without β-glucodisaccharide. The difference in cellulase activity was large in early stage of culture.

The changes in amounts of glucose, pH and cell mass showed similar tendency when *A. xylinum* ATCC23769 cultured in three different types of media (data not shown). The changes in amount of oligosaccharides containing in the medium with β-glucodisaccharides during the culture are shown in Fig. 6. In SH medium and SH medium with cellobiose, a small amount oligosaccharides are produced after 1day and began to increase gradually. Whereas the production of these oligosaccharides in SH medium with gentiobiose were not observed in the early stages. Nevertheless, the production of oligosaccharides among three kinds of media exhibited very similar profiles with each other.

The changes in cellulose production during the culture are shown in Fig. 7. The cellulose production in the case of gentiobiose added medium was the smallest among three kinds of medium in early stages. But at a later stage the production of cellulose in the medium with cellobiose and

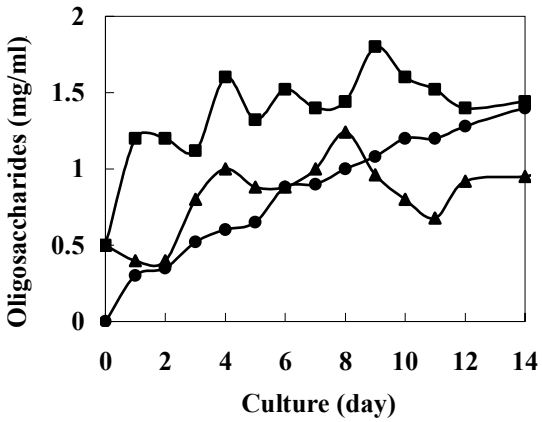

Fig. 6. Changes in the amounts of disaccharides in three kinds of culture broths of *A. xylinum* ATCC23769.
Symbols: ●, SH medium; ■, SH medium with cellobiose; ▲, SH medium with gentiobiose.

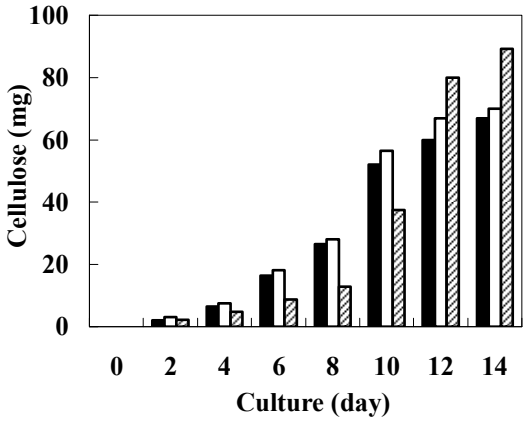

Fig. 7. Changes in cellulose production in three kinds of culture broths of *A. xylinum* ATCC23769.
Black, white and stripe bars represent SH medium, SH medium with cellobiose and gentiobiose respectively.

gentiobiose increased rapidly and amounts of cellulose produced were larger than that in normal medium after 12 days, which reached maximal at 90 mg/m*l*. When *A. xylinum* cells were grown in the medium containing β-glucodisaccharides, it seems that the production of cellulolytic enzymes is accelerated and the amounts of cellulose are increased.

From all of the above results, it can be concluded that cellulose production closely relates to cellulase activity, and that all sorts of oligosaccharides produced during *A. xylinum* culture can induce the production of cellulase and resulting to accelerate the cellulose production. Therefore, the production of cellulolytic enzymes seems to be closely related to the production of oligosaccharides, as cellulase could attack acetan in cooperation of other enzymes.

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