*J. Biol. Macromol.*, **5**(1), 3-10 (2005)

## Review

# Novel cellulose producing system by microorganisms such as *Acetobacter* sp.

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Received March 7, 2005; Accepted March 14, 2005

### *History of bacterial cellulose*

Cellulose is the most abundant natural polymer in the biosphere during human history for industrial use such as energy, materials, paper, clothes and foods. Higher plants in addition to certain microorganisms and animals mostly produce cellulose. Some strains of *Acetobacter* species produce a gelatinous membrane called a pellicle on the surface of liquid culture, and this membrane is composed of pure cellulose.

It was about one century ago that the first scientific paper was written by Brown in 1886 (1) on a peculiar fermentative substance that would have been known in many places on earth and was particularly popular in his country under the name of "vinegar plant". Under pure cultivation in carbohydrate media, it was observed that the whole surface of the liquid is covered with a gelatinous membrane, which may attain a thickness of 25 mm under favorable circumstances. On removing the membrane from the liquid, it is found to be very tough, especially if an attempt is made to tear it across its plane of growth. From chemical analysis and various reactions, the substance was concluded without doubt to be cellulose, although microscopy at that time only gave a picture of living bacteria embedded in a transparent structureless film.

During the following one hundred years, a number of studies have been made on the structure of the pellicle as well as its production. Today, it is known that the pellicle comprises a random assembly of fibrils, less than 130 nm wide, which are composed of a bundle of much finer microfibrils that are 2 to 4 nm in diameter (2, 3). It is also known that the pellicle gives a film or sheet if it is dried. The crystallographic form of this cellulose has been almost the same as that of "cellulose I," commonly found in plant cellulose, and the molecular orientation is parallel to the direction of cellulose chain axis (4, 5).

Such a peculiar super-molecular structure engineered by nature has come to be of great interest to the present author when compared with other synthetic polymers that have been created to make super-strong fibers during the last two decades. Bacterial cellulose has been found to have unique structural and physical

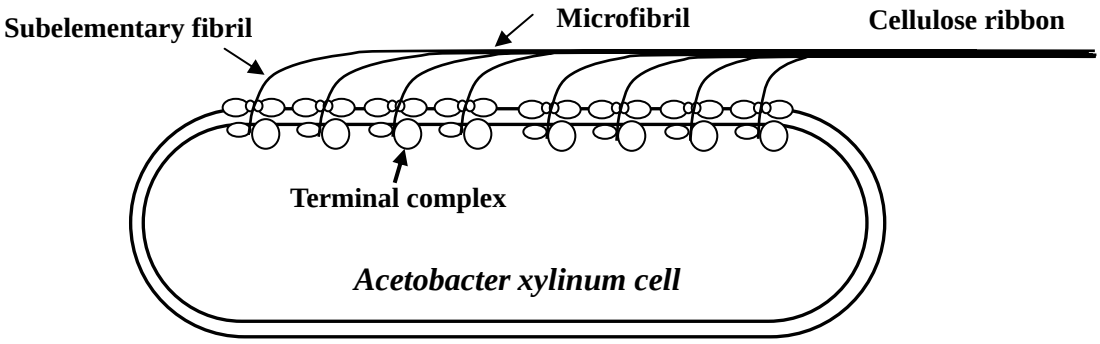


Fig.1. Schematic mechanism for cellulose ribbon formation by the aggregation of cellulose chains extruded from *Acetobacter* cell.

properties compared with higher plant cellulose; it has an extremely fine fiber network and high crystallinity, sheets of which have higher Young's moduli than those from plants. These novel physical properties would be expected to be useful in many industrial applications. To the author's knowledge, bacterial cellulose is commonly used for only indigenous desert food in the Philippines, medical pads and acoustic transducer diaphragms. Present day culture methods do not allow mass production of bacterial cellulose at low costs, which is necessary for its use as a commodity material. However, understanding of the structure and mechanical properties of bacterial cellulose is also important for industrial use.


Fig. 2. Structure of cellulose synthesizing protein gene of *Acetobacter xylinum*. egl: endo- β-1,4-glucanase, ORF2: unknown, β-gl: β-glucosidase, bcsA-D: cellulose synthase operon.

**Mechanism of cellulose biosynthesis**

Bacterial cellulose was extruded through cell membrane when several cellulose chains were synthesized by cellulose synthase that are located on inner membrane. Cellulose synthesizing protein complexes, called terminal complexes (TC), are aligned as straight line along the long axis of cell as shown in Fig.1. First, TC synthesizes several chains of cellulose, called subelementary fibril, and then several subelementary fibrils were associates and forms microfibrils, and finally they make cellulose ribbon (6). These protein complexes are coded by cellulose synthase operon, which are composed of bcsA, bcsB, bcsC and bcsD (7,8). The domain A coded by bcsA and attached inner membrane, is thought to be the catalytic domain synthesizing cellulose from UDP-glucose.

Another domain B coded by bcsB accelerated cellulose synthesis by combined to cyclic-di-GMP. It is proposed that domain C and D coded by bcs C and D might relate to the aggregation of each cellulose chain synthesized, but the primary function of these domain have been unknown.

***Cellulase from cellulose producing biology***

Cellulases catalyze the hydrolysis of cellulose and are thought to be potentially useful for the transformation of cellulose into soluble and fermentable sugars, thus providing a source of renewable energy and chemicals (9). Cellulose degradation requires a complex enzyme system composed of endo-1,4-β-glucanase, exo-cellobiohydrolase and β-glucosidase (10). Various reports have described the production of cellulases by cellulose-producing *Acetobacter* strains. Husemann and Werner (11) first reported that *Acetobacter xylinum* ATCC12733 produces a carboxymethyl-cellulose (CMC)-hydrolyzing enzyme (CMCase; endo-1,4-β-glucanase). Two different genes encoding endo-1,4-β- glucanase in different strains of *A. xylinum* were reported by two groups and the amino acid sequences encoded by these genes were entirely different in the two strains (12,13). Standal *et al*. (12) reported not only the presence of an endo-1,4-β-glucanase activity in the culture media from several cellulose-producing *Acetobacter* strains but also the location of endo-1,4-β-glucanase gene which is present upstream of the cellulose synthesizing operon of *A. xylinum* ATCC23769 (Fig.2). It is noteworthy that β-glucosidase gene is also located on the downstream of cellulose synthase operon. However the role of such indigenous cellulases in cellulose production remains unknown. Tonouchi *et al*. (14) reported that the addition of a small amount of endo-glucanase (~3 μg/mL) enhances bacterial cellulose production by *A. xylinum*. Furthermore, the addition of a large amount of β-glucosidase (100 μg/mL) from sweet almond also enhances bacterial cellulose production. Husemann and Werner (10) reported that the degree of polymerization (DP) of bacterial cellulose produced by *A. xylinum* ATCC12733 is decreased by a cellulase released into the medium during prolonged static culture. However, Marx-Figini and Pion (15) reported that the amount of cellulose with its weight-average degree of polymerization (DPw) increases linearly with cultivation time, and are proportional to the cell number in

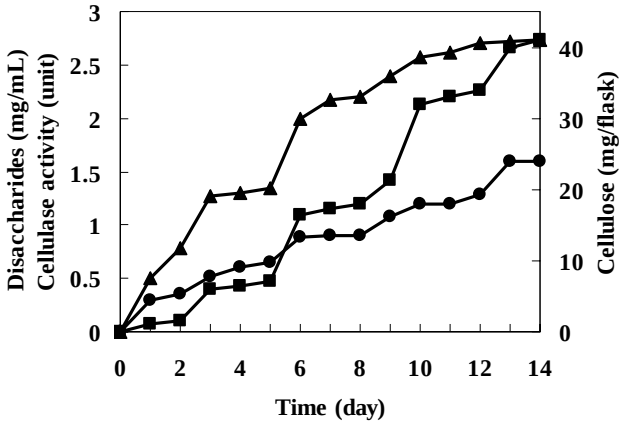


Fig. 3. Changes in cellulase activity, cellulose production and the amounts of oligosaccharides during the culture of *A. xylinum* ATCC23769.
Symbols: ●, disaccharides; ■, cellulose; ▲, cellulase activity.

shaking-culture of *A. xylinum*; at the end of cell growth, both stop changing. They suggest that the decrease in the DP of cellulose is not caused by its particular cellulase. Therefore, the factors that regulate the DP of bacterial cellulose produced by *Acetobacter* cultures is not known yet. Moreover, the relationship between the DP of bacterial cellulose and its physical properties has never been reported.

We have also investigated the production of cellulase in the culture broth of *A. xylinum* ATCC23769 (Fig. 3). 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 stage 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 (16). In addition, morphology changes in bacterial cellulose produced by *Acetobacter xylinum* ATCC23769 were observed in the presence of β-glucodisaccharides such as gentiobiose and cellobiose. Endo-β-1,4-glucanase activity in culture broth was higher than that in the absence of those sugars. From these results, we has investigated the properties of endo-β-1,4-glucanase produced by this bacteria.

***The property of cellulase from Acetobacter xylinum***

Endo-1,4-glucanase (AEG) from *A. xylinum* could hydrolyze water-soluble cellulose such as CMC, hydroxyethyl cellulose (Table 1) and cellodextrin, and decreased the viscosity of the substrate (17). On the other hand, AEG could not produce any soluble sugars from water-insoluble cellulose such as Avicel and $H_3PO_4$-swollen cotton. It is interestingly that bacterial cellulose could not degrade by own cellulase after ribbon formation which is crystalline cellulose. These properties were completely different from endo-glucanase

Table 1. Substrate specificities of endoglucanase from *A. xylinum.*

| Substrate | Specific activity ($\times 10^{-3}$ unit/mg) |
|---|---|
| **Water soluble substrate** | |
| **Carboxymethyl Cellulose** | **0.66** |
| **Xyloglucan** | **0.40** |
| **Hydroxyethyl Cellulose** | **0.68** |
| **p-Nitrophenyl Glucoside** | **1.06** |
| **Water insoluble substrate** | |
| **Avicel** | **0** |
| **$H_3PO_4$-swollen Avicel** | **0** |
| **$H_3PO_4$-swollen Cotton** | **0** |
| **Bacterial Cellulose** | **0** |

from fungi. AEG could hydrolyze cello-oligosaccharides more than DP 5 and produced cellobiose, cellotriose and cellotetraose, but when bacterial cellulose was added into the reaction mixture containing cellohexaose the hydrolysis products disappeared in the reaction mixture. It is suggested that AEG might have transglycosyl activity. As AEG is, however, inverting enzyme which belongs to glycosidase family 8, the other enzyme produced by *A. xylinum* could catalyze the transglycosyl reaction. It is proposed that this activity might be closely related to cellulose synthesis.

***Oligosaccharides produced by Acetobacter xylinum***

Some researcher have tried to investigate production of various carbohydrates and cellulose production in the culture broth of the cellulose producing microorganism *Acetobacter xylinum*. Various oligosaccharides, in addition to cellulose, have already been detected after one-day culture of this microorganism. These sugars and oligosaccharides increased gradually during the culture (Fig. 3) and 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 (18). Valla *et al.* (19) also detected mannose, rhamnose, glucuronic acid, glucuronyl mannnose and disaccharide consisting of two glucose monomers in culture broth of *A. xylinum*. They assumed this disaccharide was probably gentiobiose, though the origin of these sugars was not identified. HPLC analysis by ABEE-conversion of β-glucodisaccharides showed the detection of disaccharides such as gentiobiose, cellobiose and a very little laminaribiose. Moreover hydrolysis products from these oligosaccharides by β-glucosidase were mainly glucose (data not shown). It is reported that some strain of *A. xylinum* also produces soluble polysaccharide, called acetan (20) as shown in Fig.5. It is assumed that these oligosaccharides may be enzymatic degradation products of acetan produced simultaneously in the culture broth since the cellulase produced by this microorganism could not degrade bacterial cellulose produced by itself. It is proposed that cellulase activity is correlated closely with

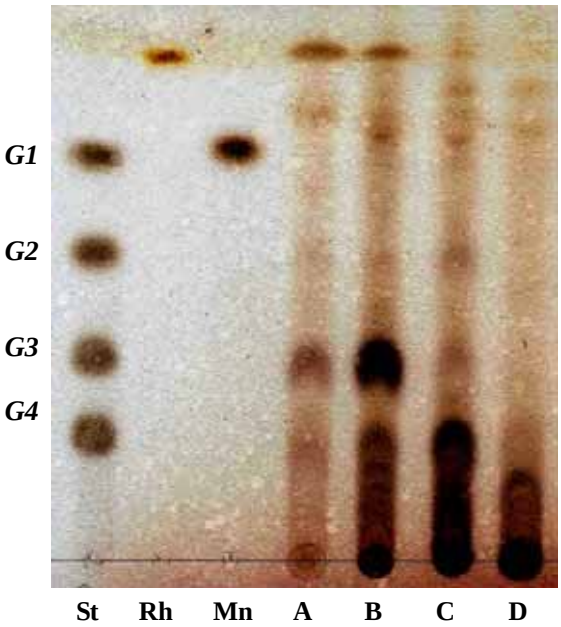


Fig. 4. Thin layer chromatogram of oligosaccharides produced by *A. xylinum*. Symbols: S1, standard sugars; G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; Rh, rhamnose; Mn, mannose. A～D: fractions eluted on charcoal column chromatography with various solvent as follows. A, 5 % EtOH; B, 10 % EtOH; C, 15 % EtOH; D, 20 % EtOH.

cellulose production, and more closely related to the changes in the 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. It is suggested that those oligosaccharides are the degradation products from acetan, as enzymes prepared from culture broth could hydrolyze acetan rather than cellulose.

***Future study***

The effects of β-glucodisaccharides additives, such as cellobiose and gentiobiose, on bacterial cellulose (BC) produced by *A. xylinum* ATCC23769 have been examined. The assembly of microfibrils in the bacterial cellulose was altered in the presence of these additives.

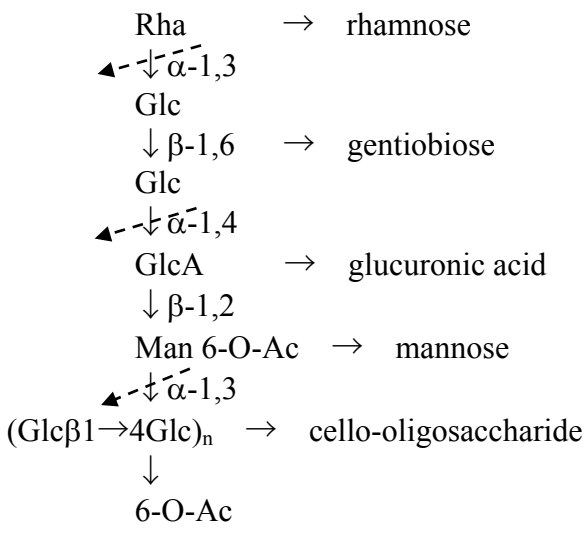


Fig.5. Structure of acetan and its constituent sugars produced by *A. xylinum.*

Bacterial cellulose produced in SH medium containing cellobiose had larger DPw/DPn values than that produced in the control medium. On the other hand, BC produced in SH medium containing gentiobiose (BC-G) had smaller DPw/DPn values. The Young's modulus of BC sheets made of BC-G was about 1.6 times greater than that of normal BC sheets. It is confirmed that the assembly of thinner microfibrils result in the production of stronger BC sheets. Previously we reported that CMC-degrading activity in the presence of β-glucodisaccharides additives was higher than that in the normal medium during early stages of growth (21). Although further studies are required to clarity the effect of additives, it is proposed that CMC-degrading activity is closely related to synthesis and modification of bacterial cellulose. It is noteworthy that most of cellulose producing organism produced cellulose degrading enzyme commomly. Therefore the role of this enzyme for cellulose synthesis expects to be clear.

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Communicated by Hiroshi Ueno