Effects of Oxygen Tension in the Gaseous Phase on Production and Physical Properties of Bacterial Cellulose Formed under Static Culture Conditions†

Kunihiko Watanabe†† and Shigeru Yamanaka

Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan
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Acetobacter produces a gelatinous membrane composed of a cellulose network when grown in liquid culture under static conditions. The oxygen tension in the gaseous phase was found to have an effect on cellulose production and on the physical properties of the membrane. Cellulose production was higher at oxygen tensions of 10% and 15% than that under atmospheric conditions. In contrast, cell growth remained constant under a variety of oxygen tensions. Unexpectedly, the density of the cellulose network was found to be inversely proportional to the level of cellulose production by cells in the membrane through electron microscopic examination and other studies. It was concluded that this interesting phenomenon can be explained by our previously proposed model of BC network formation.

Some strains of Acetobacter produce cellulose (bacterial cellulose) as a gelatinous membrane on the surface of cultures grown under static conditions. We found that a specific sheet having a very high Young's modulus can be prepared from bacterial cellulose (BC) membrane. This material is now being used as a transducer diaphragm. In addition, the application of BC to wound dressing and as a carrier for mammalian cell culture has been attempted on the basis of its high water permeability and mechanical strength when moist, as a result of the network structure of the cellulose fibrils forming the membrane.

Many scientists have shown interest in BC as a model of cellulose biogenesis. Brown first reported the fermentative production of BC. Hestrin, Schramm, and Dudman extensively investigated the optimum fermentative conditions for producing BC in the 1950s. Authors of these reports mainly dealt with studies on static culture. Acetobacter species are obligate aerobes. It is thought that these organisms produce cellulose at the air-liquid interface, with the cells being located on the surface of the culture so as to obtain oxygen. Hestrin et al. suggested that the higher oxygen tension in the gaseous phase than in the atmosphere was more suitable for cellulose production. In this study, we examined the effects of changes in the oxygen tension in the gaseous phase on cellulose production and the properties of the membrane.

Materials and Methods

Microorganisms. Acetobacter aceti subsp. xylinum AJ12725 was used unless otherwise specified. Acetobacter sp. AJ12712, AJ12726, Acetobacter xylinum ATCC10821, ATCC23769 were also used.

Culture medium. The medium was: sucrose, 50 g/liter; yeast extract, 5 g/liter; (NH₄)₂SO₄, 5 g/liter; KH₂PO₄, 3 g/liter; and MgSO₄·7H₂O, 0.5 g/liter. The pH was adjusted to 5.0 with NaOH or HCl.

Culture. One-tenth of a milliliter of frozen stock culture was inoculated into 10 ml of culture medium in a test tube with an inner diameter of 16 mm and culture was done for 1 week under static conditions. The resulting seed culture was shaken vigorously to release cells from the gelatinous membrane, and 2 ml of the cell suspension thus obtained was inoculated into 20 ml of the same medium in an open vial with an inner diameter of 30 mm. This culture was incubated at 30°C for 1 week in a sealed vessel with a volume of 12 liters under a controlled oxygen tension.

Analytical procedures.

(1) Cellulose content. The gelatinous cellulose membrane produced on the surface of the cultures was picked up with tweezers and weighed to measure the wet weight. After the membrane was washed in water, it was pressed while being heated at 120°C for 5 min and then the dry weight of the membrane was measured. To eliminate cells and purify the cellulose, the dried membrane was soaked in 0.5 N NaOH for 20 min at 100°C and washed in running water. The cellulose was then weighed after drying the membrane at 105°C until a constant weight was obtained.

(2) Cell content. The cell content of the BC membrane was calculated as the difference in weight between the dried membrane after just washing in water and that after hot caustic soda treatment.

(3) Residual sugar and gluconic acid. The residual levels of sugar and gluconic acid (as the sum of gluconic acid and 2 keto gluconic acid) in the culture broth were measured by HPLC using a Sugar SP1010 (Shodex, Japan) column and KC811 (Shodex) column, respectively.

Results

Effects of oxygen tension in the gaseous phase on BC production and cell growth

BC production and cell growth were measured at various oxygen tensions. Figure 1 shows the effects of various oxygen tensions in the gaseous phase on cellulose production and cell growth. In this report, oxygen tension is expressed as the volume % in the gaseous phase at 1 atm. A higher oxygen tension in the gaseous phase than in the atmospheric tension was found to inhibit cellulose production. This result supports our previous finding that the thickness of the gelatinous membrane decreased with elevation of the oxygen tension higher than that in the atmosphere.

On the other hand, an oxygen tension lower than atmospheric (10–15%) led to about 25% higher cellulose production. In contrast, the influence of oxygen on cell growth was minimal compared with its effect on BC production.

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†† Corresponding author. Present affiliation and address: Bio-polymer Research Co., Ltd., KSP R&D B-1015, 3-2-1 Sakato, Takatsu-ku, Kawasaki 213, Japan.

Abbreviation: BC, bacterial cellulose.
Hestrin et al.\textsuperscript{6} reported that BC production from glucose was about half-maximal at 20% oxygen and gradually approached maximum at 100% oxygen during agitated culture of freeze-dried \textit{Acetobacter xylinum} cells for 1–3 h. The discrepancy between our results and those of Hestrin et al.\textsuperscript{6} may be due to differences in the duration of culture or the growth phase of the cells.

Figure 2 demonstrates yield coefficients (\%, grams product produced/grams sucrose consumed) for cellulose, cells, and gluconic acid. The highest yield coefficient for cellulose was observed at a low oxygen tension (10\%). Among the 4 strains examined, it was observed that a low oxygen tension of 10\% was the most suitable for cellulose production (Table I). In contrast, the yield coefficients for cells and gluconic acid remained constant despite changes in the oxygen tension (Fig. 2). The CO\textsubscript{2} tension was also measured and the yield coefficient was calculated (Table II). The yield coefficients of CO\textsubscript{2} (\%, grams CO\textsubscript{2}/grams sucrose consumed) at the oxygen tensions of 10 and 50\% was 69 and 106\%, respectively. Thus, bacterial respiration was stimulated by a higher oxygen tension, while the cellulose production was inhibited.

Although the metabolic pathway for cellulose synthesis and its regulatory system have been extensively investigated,\textsuperscript{11} no biochemical effect of oxygen on cellulose synthesis has been reported previously. To analyze the biochemical effects of oxygen on cellulose production, the situation for other extracellular polysaccharides was reviewed. It was found that Thompson \textit{et al.}\textsuperscript{12} reported that extracellular soluble polysaccharide production by \textit{Rhizobium trifolii} increased and cell growth decreased under low oxygen conditions, although they did not refer to the mechanism.

Effects of oxygen on the physical properties of BC membrane

The thickness of the membrane decreased with an increase in oxygen tension (Fig. 3). Also, the texture of the gelatinous membrane under higher oxygen tensions than atmospheric became harder than that under atmospheric conditions, while a softer membrane was produced at lower oxygen tensions.

As expected from the texture of the membrane, its cellulose content increased with an increase of the oxygen tension in the gaseous phase (Fig. 3).

To analyze these phenomena, we observed the structure of BC membranes produced at various oxygen tensions using the scanning electron microscopy. Figures 4A, 4B, and 4C show the micrographs of membranes which were produced at oxygen tensions of 10\%, 21\% (air), and 50\% oxygen, respectively. The membrane produced at the highest oxygen tension (50\%) had a denser network of cellulose fibrils than that at the lowest oxygen tension (10\%). There was, however, no apparent difference in the width of the fibrils among these membranes even at a higher magnification. Judging from these results, the BC content of the gelatinous membrane does not reflect the width of the individual fibrils but instead reflects the density of the network.

### Table I. BC Yield Coefficients from Sucrose in 4 Strains of \textit{Acetobacter} at 10\% Oxygen and 21\% Oxygen under Static Culture Conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>BC yield coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% O\textsubscript{2}</td>
</tr>
<tr>
<td>AJ 12725</td>
<td>16.0</td>
</tr>
<tr>
<td>AJ 12712</td>
<td>22.1</td>
</tr>
<tr>
<td>AJ 12726</td>
<td>14.1</td>
</tr>
<tr>
<td>ATCC 10821</td>
<td>11.6</td>
</tr>
<tr>
<td>ATCC 23769</td>
<td>14.2</td>
</tr>
</tbody>
</table>

### Table II. CO\textsubscript{2} Production from Sucrose at 10\% and 50\% Oxygen

<table>
<thead>
<tr>
<th>Oxygen conc.</th>
<th>CO\textsubscript{2} tension after culture (%)</th>
<th>CO\textsubscript{2} yield coefficient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>1.1</td>
<td>69</td>
</tr>
<tr>
<td>50%</td>
<td>1.7</td>
<td>106</td>
</tr>
</tbody>
</table>

* Grams CO\textsubscript{2} produced/grams sucrose consumed.
Effects of Oxygen on Bacterial Cellulose Production

Discussion
As described above, the oxygen tension in the gaseous phase under static culture conditions affected both BC production and the physical properties of the membrane, such as the density of the cellulose network and membrane toughness. We analyzed the relationship between cellulose production (calculated from the data in Fig. 1), and the cellulose content of the gelatinous membrane (Fig. 3). As shown in Fig. 5, there was a distinct negative correlation between the level of cellulose production and the cellulose content of the membrane. This means that the density of the network decreases with an increase of cellulose production by the bacteria (grams BC/grams cells).

We consider that this interesting phenomenon can be explained as follows by our previously proposed model of BC network formation. According to our model, cellulose fibrils branch in association with cell division, as illustrated in Fig. 6, and these branches produce a ramiform network. The length of the segment between two branches appears to be related to the density of the network.

We calculated the lengths of the segments between branches in cellulose networks formed at oxygen tensions of 10% and 50%. In this model, the cells exist at the ends of BC fibrils in the ramiform network. On the basis of our observations, the average width of the cellulose fibrils produced by *Acetobacter* remains almost constant during the entire culture period. Therefore, the weight of the cellulose must reflect the total length of BC fibrils, which can be calculated as follows:

\[ T_L = \frac{W}{DA} \]  

where:
- \( T_L \): Total length of BC fibrils produced
- \( W \): Weight of cellulose
- \( D \): Density of cellulose
- \( A \): Cross-sectional area of a single BC fibril

The density of cellulose was assumed to be 1.59 according to data reported by Sugiyama et al. The cross-sectional area of a single cellulose fibril was assumed to be \( 4 \times 80 = 320 \text{nm}^2 \) according to the results of Zaart.

The number of segments between two branches can be obtained as follows:

\[ S = N_0 + 2N_0 + 4N_0 + \cdots + 2^{n-2}N_0 + 2^{n-1}N_0 + 2^nN_0 \]
$S$: Number of segments between branches
$N_0$: Number of bacteria at inoculation
$n$: Number of generations during the entire culture period
In addition the number of cells at the end of culture can be calculated as follows:

$$N = 2^n N_0 .$$  \hspace{1cm} (3)

$N$: Number of cells at the end of culture
The number of cells was estimated from the dried cell weight and by direct counting of an optical microscope after digestion of the BC membrane with cellulase.
Therefore, Eq. (2) can be rewritten as follows

$$S = N + N/2 + N/4 + \cdots$$
$$+ N/2^{(n-2)} + N/2^{(n-1)} + N/2^n \approx 2N .$$  \hspace{1cm} (4)

Thus, the length of a segment can be calculated as follows:

$$L = T_L/S = T_L/2N .$$  \hspace{1cm} (5)

$L$: Length of the segment between two branches
Thus, the length of a segment between two branches on a cellulose fibril produced at 10% and 50% oxygen was calculated as about 700 \( \mu m \) and about 200 \( \mu m \), respectively, as indicated in Fig. 6. Such differences in length seem to be related to differences in the density of the network, as shown in Fig. 4.

Brown et al.\(^{15}\) and Kai et al.\(^{16}\) independently reported that a single fibril of cellulose was excreted at a rate of about 2 \( \mu m \) per minute by cells in a static culture on a grid for TEM specimens. In our study, the doubling time of the bacteria was estimated to be 8 h (data not shown), and this value agrees with the longest doubling time for Acetobacter reported by Figinin et al.\(^{17,18}\) (1.5–8 hours under agitated culture conditions). Since the length of a segment calculated using these data ranged from 180 \( \mu m \) to 960 \( \mu m \), our range of about 200–700 \( \mu m \) is considered to be reasonable. However, it would be very difficult to directly observe a single part of the ramiform network by electron microscopy because the gelatinous membrane is composed of such a complex network and because the length of a segment is too long to fit in the field of view of the electron microscope.

In this study, we examined the effect of oxygen tension on network formation and our findings suggest that the density of the network in the gelatinous membrane can be controlled.

The density of the cellulose network may be related to membrane toughness. In addition, the density of the cellulose network may be correlated with water permeability. Controlling such properties by altering the oxygen tension may be useful in producing membranes for various applications, such as wound dressing\(^3\) and a carrier for mammalian cell culture.\(^4\) Our findings may assist in achieving the biotechnological control of BC properties which depend on the network structure.

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References