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The Influence of Culture Conditions on the Fibrillation of Cellulose Gel Produced by *Acetobacter xylinum*

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The influence of the culture conditions, such as the temperature of incubation and the pH of the culture medium, on the fibrillation of cellulose gels produced by *Acetobacter xylinum* was studied by means of electron microscopy. The incubation temperature did not show any influence on the fibrillation of cellulose gels as long as it was in the range between 10 to 28 °C. However, in the incubation at 5 °C the fibrillation occurred very slowly. When incubation was carried out at 5 °C, the fibril produced by the 5 °C incubation maintained an amorphous state for about 3h, and the microscopic fibrils appeared in the fibril only after incubation for 12h. However, the width of the microscopic fibril was not influenced by the temperature of incubation. The pH of the medium influenced the linear growth rate of a fibril. The rate increased with an increase in the pH, reached the maximum at pH 6.8, and decreased at higher pHs. The fibrillation occurred more rapidly at a constant temperature as the linear growth rate was lowered, but the width of a microscopic fibril was not influenced by the pH of the medium, just as it was not influenced by the temperature.

The fibril just after production by *Acetobacter xylinum* (*A. xylinum*) seems to be in a homogeneous, amorphous state.[1,2] However, When the amorphous fibril is extruded from the cell, it becomes clearer that its cellulose chains are already arranged as sheets of monomolecular layers corresponding to the ($1\bar{1}0$) plane of Cellulose I.[3,4] In the amorphous fibril regarded as an assembly of the cellulose sheets, microscopic fibrils with almost the same width (about 40 Å) were always developed in the course of time.[5,6] It is interesting to study whether the width of a microscopic fibril is dependent on the culture conditions, that is, the crystallization conditions, or whether it is dependent on the fact that, when cellulose is extruded from the synthesizing sites in the envelope of a bacterial cell,as reported by Brown *et al.*[7] and Zaar,[8] the precursors of microscopic fibrils are already included in it.

In this paper the influence of the culture conditions, such as the incubation temperature and the pH of the medium, on the fibrillation of cellulose gels produced by *A. xylinum* was studied by means of electron microscopy.

Experimental

Culture of Cells and Preparation of the Samples for Electron Microscopy. The methods described previously[3] were employed.

Preparation of Cellulose-free Cell Suspension: An amount of 10 ml of an *A. xylinum*-preserved culture medium (IFO 13693) was added to 100 ml of an complex medium, and the mixture was incubated at 28 °C for 72 h. An amount of 10 ml of the liquid phase of the starter culture was added to 100 ml of the fresh complex medium, and the mixture was incubated at 28 °C for 48 h. The cells collected from the incubated medium according to the method of previous papers[1,3] were suspended in 60 ml of a buffer solution with a pH value of 6.8.

Samples for the Study of the Influence of the Temperature of Incubation: A 4-ml quantity of a 0.2 wt% glucose medium with 2 wt% sodium tungstophosphate (pH 6.8) was added to 36 ml of a cell suspension. A drop of the culture medium was immediately placed on a sheet mesh covered with a collodion membrane and incubated in a 100% RH atmosphere at a given temperature for a given time. After the fibrils thus produced had been allowed to settle on the sheet mesh by removing the medium, they were dried over P_2O_5 for more than 24 h at room temperature and then observed through an electron microscope.

Samples for the Study of the Influence of the pH of a Medium: After the cell suspension and the 0.2 wt% glucose medium with 2 wt% sodium tungstophosphate had been adjusted to a given pH with 1 M† HCl or NaOHaq respectively, a 4-ml quantity of the glucose medium was added to 36 ml of the cell suspension. A drop of the culture medium was placed on a sheet mesh and incubated in a 100% RH atmosphere at 28 °C for a given time. After this incubation, samples for electron microscopy were prepared as has been described above.

Measurement of the Linear Growth Rate of a Fibril. The linear growth rate of a fibril under a given pH was obtained from the relation between the time of incubation and the mean length of ten fibrils after glucose incubation. The linear growth rate was constant within the range of the measureable length of a fibril.

For electron microscopy, all the preparations were examined with a JEOL JEM-100U electron microscope operating at 80 kV.

Results and Discussion

Brown *et al.*[9] suggested that the rate of crystallization

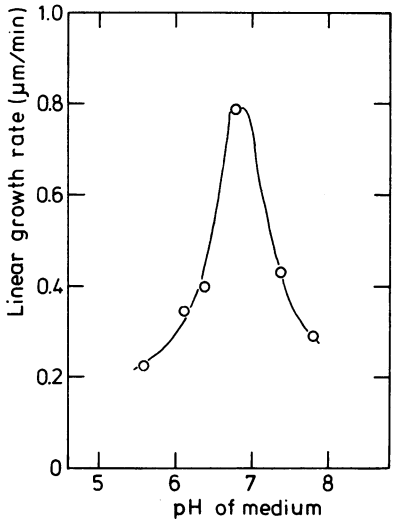

Fig. 1. Influence of the pH of medium on the linear growth rate of a fibril. The temperature of incubation; 15 °C.

† 1 M=1 mol dm^{-3}.

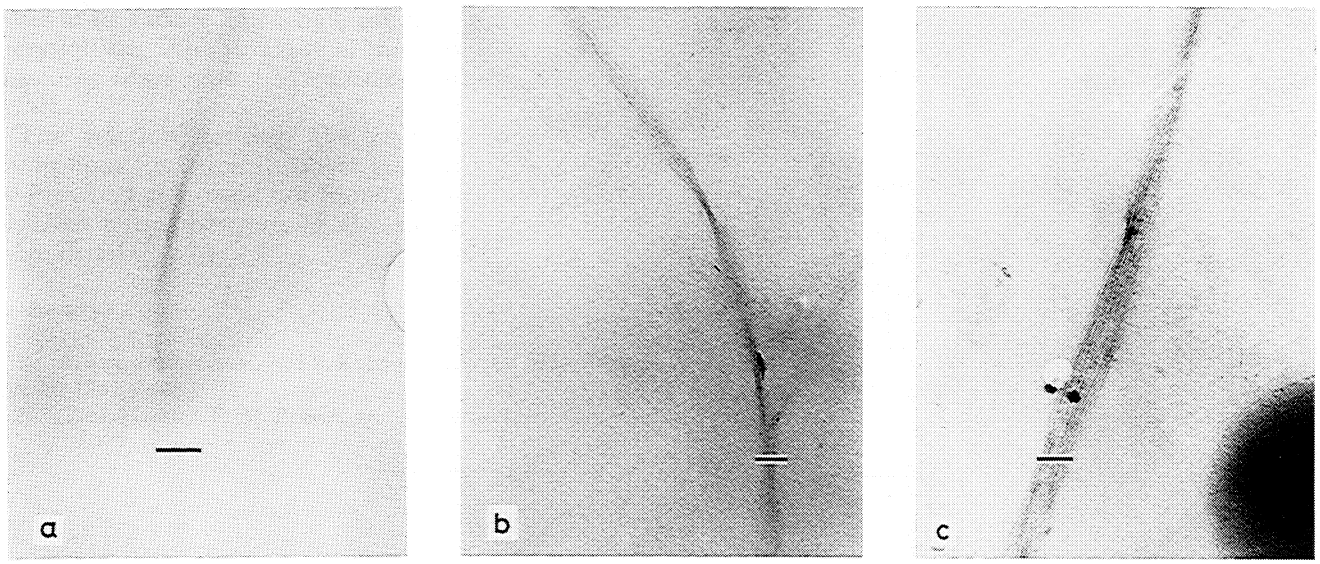

Fig. 2. Electron micrographs of the fibrils from the 5 °C incubation (pH 6.8) for a given time. Time of incubation; a: 3 h, b: 6 h, c: 12 h. Scale bar=0.1 μm

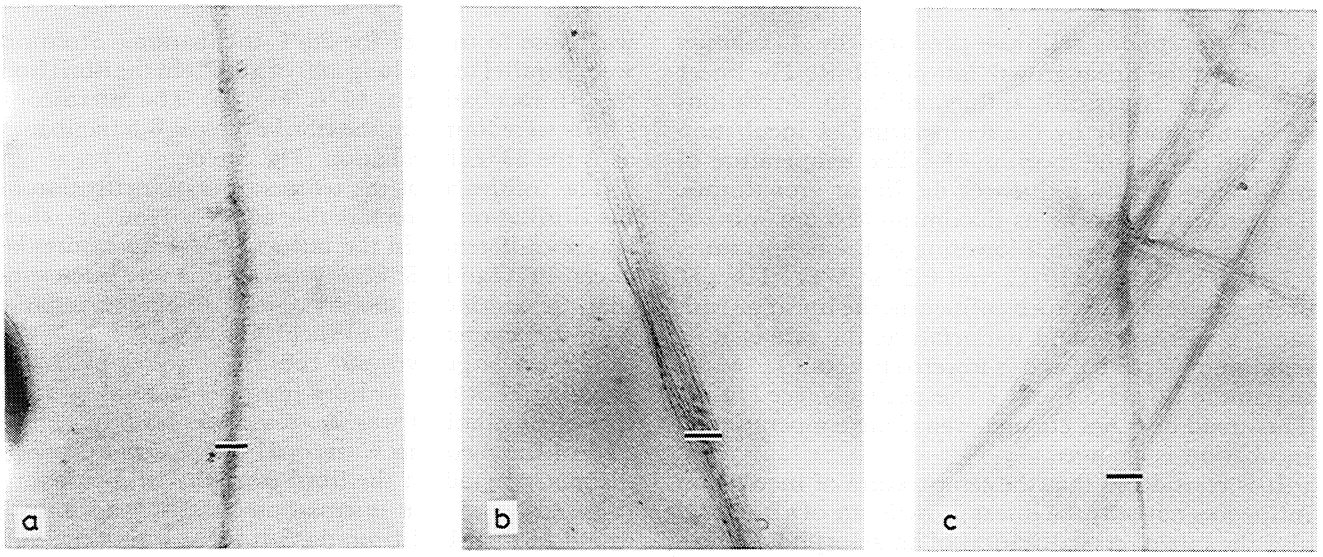

Fig. 3. Electron micrographs of the fibrils from the 10 °C incubation (pH 6.8) for a given time. Time of incubation; a: 3 h, b and c: 6 h. Scale bar=0.1 μm

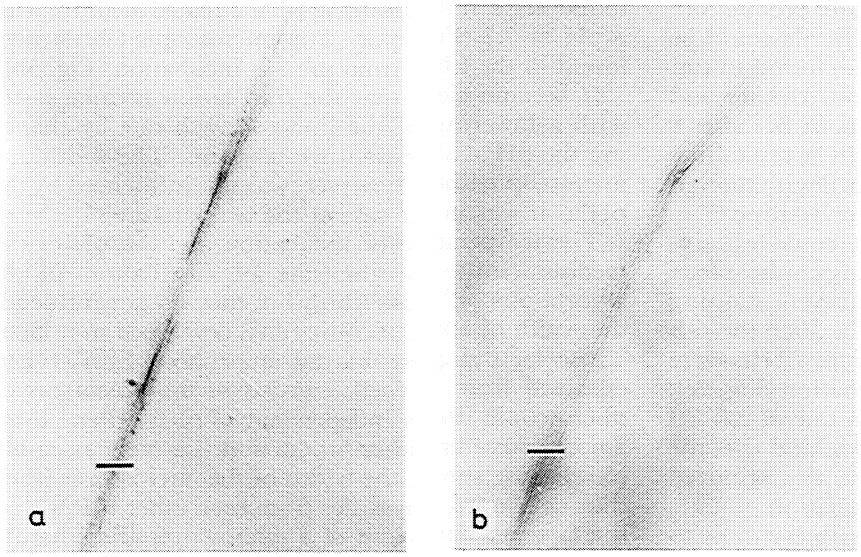

Fig. 4. Electron micrographs of the fibrils from the incubated medium of pH 5.5 at 28 °C for a given time. Time of incubation; a: 1 h, b: 3 h. Scale bar= 0.1 μm

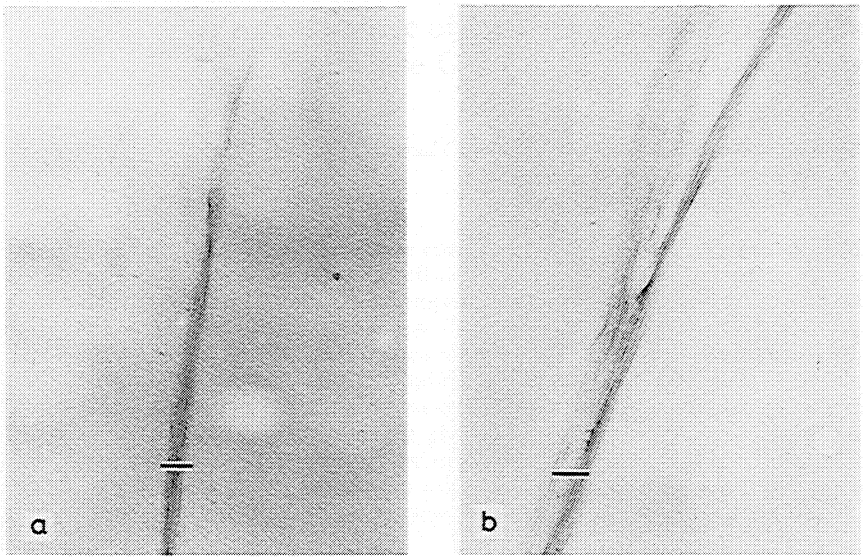

Fig. 5. Electron micrographs of the fibrils from the incubated medium of pH 7.7 at 28°C for a given time. Time of incubation; a: 1 h, b: 3 h. Scale bar= 0.1 μm

of the amorphous cellulose produced by *A. xylinum* influences the linear growth rate of a fibril. The linear growth rate is basically influenced by the conditions of culture, mostly by the temperature of incubation and the pH of the medium.[3,6] The temperature of incubation greatly influences the linear growth rate. The growth rate is at its maximum (2.6 μm/min) at 28°C and lowers with a lowering of the temperature: 1.3 at 20°C, 0.39 at 10°C, and 0.27 at 5°C. The pH of the medium also much influences the linear growth rate. Under a constant temperature, the rate is at its maximum at pH 6.8 and is lowered to about 1/4 of the maximum at pH 5.5 and 7.7 respectively (see Fig. 1). This trend does not change even if the temperature of incubation is changed.

It is considered that the temperature of incubation influences not only the linear growth rate of a fibril but also the rate of crystallization of an amorphous fibril. The fibril made by the incubation under the most suitable culture conditions (28°C, pH 6.8) maintained an amorphous state for about 30 min after production. The uneven staining behavior of the fibrils could be observed about 1 h later, striations were found to run parallel to the long axis of the fibril about 3 h later, and microscopic fibrils were developed about 5 h after.[6] The microscopic fibril was already in a crystalline state.[10] The resistance to alkali of a fibril with uneven staining behavior showed Cellulose I crystalline.[10]

The fibrils made by incubation at 5°C could be stained homogeneously with sodium tungstophosphate over a long period (Fig. 2a). An uneven staining behavior of the fibril was found only after the 6-h incubation (Fig. 2b), and the microscopic fibrils were found in the fibril from the 12-h incubation (Fig. 2c). It became clear that the linear growth rate of a fibril became lower under the 5°C incubation and that the fibrillation of an amorphous cellulose also occurred more slowly than under the 28°C incubation.

Although the linear growth rate of a fibril is close to that of the 5°C incubation under the 10°C incubation, the rate of the fibrillation of an amorphous cellulose was close to that of the 28°C incubation. Striations were parallel to the long axis of a fibril in the fibril from the 3-h incubation at 10°C (Fig. 3a). The microscopic fibrils were found in the fibril from the 6-h incubation as is the 28°C incubation (Fig. 3b, c).

The influence of the temperature of incubation on the fibrillation is remarkable at 5°C. However, there was no difference in the influence at temperatures between 10°C and 28°C. Moreover, it is very interesting that the width of a microscopic fibril does not change with the temperature of incubation.

The linear growth rate could be lowered by changing the pH of the medium from pH 6.8, however, the rate of fibrillation was increased accordingly. In the case of the medium with a pH of 5.5, striations were distinctly found in the fibril from the 1-h incubation (Fig. 4a), and the microscopic fibrils were already found in the fibril from the 3-h incubation (Fig. 4b).

In the case of pH 7.7, the linear growth rate is near to that in the case of pH 5.5. Striations were also clearly found in the fibril from the 1-h incubated medium with a pH of 7.7 (Fig. 5a). The microscopic fibrils were found in the fibril from the 3-h incubation (Fig. 5b).

It became clear that the fibrillation became faster with a lowering of the linear growth rate by changing the pH at a constant temperature. The width of a microscopic fibril, however, was not influenced by the pH as it was in the case of the temperature.

The temperature of incubation and the pH of the medium influence the rate of fibrillation of an amorphous cellulose fibril, but they do not influence the width of a microscopic fibril developed in it later. These facts suggest that an amorphous fibril, when extruded from the cell, consists of the precursors of microscopic fibrils independent of each other. If an amorphous fibril after production is a mere assembly of cellulose sheets, the width of the microscopic fibril thus developed may differ from each other. However, the fact that the width of a microscopic fibril hardly changes suggests that the precursors of microscopic fibrils have structures which are able to join with each

other at its surface, but which can not gather to form a crystalline. The structure of the precursor of a microscopic fibril must be hereafter examined in more detail.

The relation between the crystallization of an amorphous fibril and its linear growth rate suggested by Brown *et al.*[9] could not found in this study. This problem must, therefore, be also studied hereafter in more detail.

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