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# Kinetic aspects of bacterial cellulose formation in *nata-de-coco* culture system

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**Abstract**

The process of cellulose formation in a *nata-de-coco* culture system has been investigated. The medium was prepared with coconut-water by adding sugar and N-compounds and the culture was conducted in static conditions. The growth of gel thickness, wet weight and dry weight was almost independent of the concentrations of N-compound and sugar at least when they were above 0.1 and 1%, respectively, suggesting that the process was controlled by oxygen supply. Glucose was the only saccharide found in the coconut water stored for 3 days. While the concentration of sugar dropped quickly to a certain level and decreased monotonically with time, a part of glucose turned to something other than cellulose. No fructose was found after the initial stage, presumably being consumed by side-reactions. Computer simulation showed that, after the induction time, the process of cellulose formation or the consumption of glucose is controlled by the diffusion of atmospheric oxygen. 

*Keywords:* Cellulose formation; *Nata-de-coco* system

## 1. Introduction

Ever since its unique physical properties were discovered in mid-1980s (Iguchi et al., 1988; Yamanaka et al., 1989; Nishi et al., 1990; Iguchi, Yamanaka, Watanabe, Nishi & Uryu, 1991; Iguchi & Yamanaka, 1997), interest has grown in bacterial cellulose which is traditionally of use for *nata-de-coco*, an indigenous food of South-East Asia. Unlike ordinary cellulose from vegetable cell-walls, bacterial cellulose is generated by a species of bacteria, viz. *Acetobacter xylinum*, as an ex-cellular product in the form of a swollen pellicle. The gel-like substance comprises fine microfibrils and the peculiar morphology is considered to impart extraordinary mechanical strength, when processed into a film or sheet, that is unrivalled by artificial polymer films (Nishi et al., 1990).

The growth of pellicle was observed as early as in 1880s by Brown (Brown, 1886a,b) who identified the product as chemically equivalent to cell-wall cellulose. Experiments to trace the yield of cellulose as well as the thickness of gel were carried out by several groups (Borzani & de Souza, 1995; Yamanaka et al., 1989; Masaoka, Ohe & Sakota, 1993. It is understood that, after the initial stage, the cellulose is generated only in the vicinity of surface, because the bacterium is an aerobic one. As long as the system is kept unshaken, the disc-shaped gel is suspended by the cohesion to the interior wall of vessel and slides steadily downwards as it thickens.

With the aim of enhancing the productivity, production of cellulose has been investigated in agitated conditions in which the product is obtained in the form of slurry or small fragments (Yoshinaga, Tonouchi & Watanabe, 1997). Such conditions are also preferred for studying the kinetics, because the factor of oxygen supply can be diminished (Brown, 1962; Marx-Figini & Pion, 1974, 1976; Ring, 1982). Most of these studies used culture media prepared with pure ingredients, and fermentation was also studied with various saccharides other than sucrose (e.g. Masaoka et al., 1993). Nevertheless, static culture with natural media, viz. coconut-water obtained as a waste from the copra process, is important especially in tropical countries, even though the technique relies much on industrial experience.

The present study has been intended to study the formation of bacterial cellulose in coconut-water, with respect to the addition of sucrose (sugar) and nitrogen-containing compounds, which is a common practice in *nata-de-coco* manufacture. The change in carbohydrate concentration during culture as well as the yield of cellulose has been

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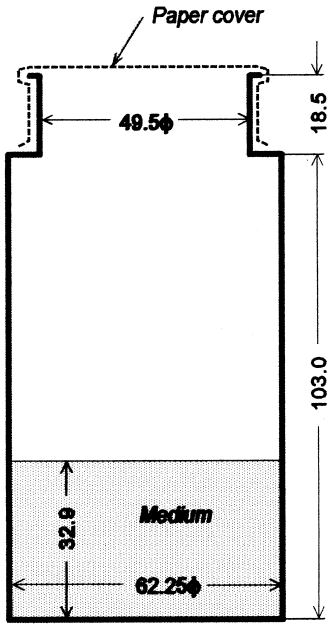


Fig. 1. Sketch of culture vessel. Unit: mm.

followed and properties of the products have been examined to discuss the mechanism and kinetics of cellulose formation. The process of cellulose formation was modelled on the assumption that it was controlled by oxygen diffusing from air.

## 2. Experimental

### 2.1. Culture medium

Following common industrial procedure, coconut-water obtained from matured fruits and stored for 3 days was used in most experiments, after sterilising in an autoclave. Sucrose added was food-grade white sugar. Ammonium sulphate, $(NH_4)_2SO_4$ (chemical-grade, Petro Kimia) and diammonium hydrogen phosphate, $(NH_4)_2HPO_4$ (chemical-grade, Merck) were chosen as nitrogen-source compounds and dissolved in the sterilised coconut-water. The acidity was adjusted to pH = 4.5 by adding acetic acid. A droplet (ca. 0.05 ml) of vitamin B syrup (from Merck, containing 2.5 mg $B_1$, 1.0 mg $B_2$, 10 mg $B_3$, 1.5 mg $B_5$, 1.0 mg $B_6$, 2 μg $B_{12}$ and 0.125 mg D( + )biotin in 1 ml) was added per litre of medium.

### 2.2. Culture method

*Acetobacter xylinum* used was a stock supply from the Institute for Research and Development of Agro-Based Industries (IRDABI), i.e. a two-decade descendant of a strain originally supplied from Philippines courtesy of the National Institute of Science and Technology, Manila.

Seeding was conducted by the two-step method. The seed broth was prepared by inoculating the bacterium in a 500 ml portion of culture medium and placing it in an incubator at 28°C for 7 days. After removing the pellicle appearing on the surface, the broth was added to the rest of culture medium in a ratio of 100 ml/1000 ml. One hundred ml portions of activated medium were distributed in glass vessels of 62.65 mm inner-diameter (see Fig. 1), covered by a porous paper and kept at 28°C for various periods, i.e. 3–14 days.

### 2.3. Measurements

After harvesting the gel from the vessel and wiping-off excess liquid, the wet-weight and the thickness were measured, the latter by means of a dial-gauge calibrated in 0.1 mm. The remaining solution was thermally sterilised without loss of time for the analysis of saccharides, i.e. sucrose, glucose, fructose etc. A high-speed liquid chromatography apparatus, Shimadzu LC-10AD equipped with an Asahipak $NH_2P$-50 column was employed for this purpose and run with 70/30 mixture of acetonitrile (Merck Lichrosol) and water at the flow rate of 1 ml/min. Standard saccharides (fructose, D(+)glucose, sucrose) used for calibration were analytical-grade products from Wako Pure Chemical.

The gel volume compressibility, $v$, was defined by

$$v = (1 - 1/r) \times 100(\%)$$

where $r$ is the ratio of the weight of gel measured before and after loading 2000 g weight on the flat surface for 30 s.

Prior to the dry weight measurement, the gel was washed with water, immersed in 0.1% NaOH solution overnight to dissolve bacteria, followed by 0.1% NaOCl solution overnight to decompose proteinaceous impurities, washed again with water and finally heat-pressed at 115°C for 5 min under 130 kg/cm$^2$. Chemicals used for processing were obtained from Soda Waru, Indonesia.

### 2.4. Calculation of cellulose production-time trace

Numerical calculation on kinetic equations assumed (Eqs. (3)–(10)) was carried out on a computer software, Corel Quattro Pro 8 (see Section 4).

## 3. Results

For two series of culture experiments carried out with different nitrogen-source compounds and different added-sugar concentrations, the changes with time of gel thickness, wet weight and dry weight, compressibility and solid content are plotted in Fig. 2. For all these parameters, a general trend was observed in that the values increased more or less linearly, after an initial period of ca. 3 days, until the rate tended to slow down after 10 days, except that the compressibility approached a constant value after ca. 6 days. Some curves of runs appears to have risen from time zero but, in reality, no gel has been observed before

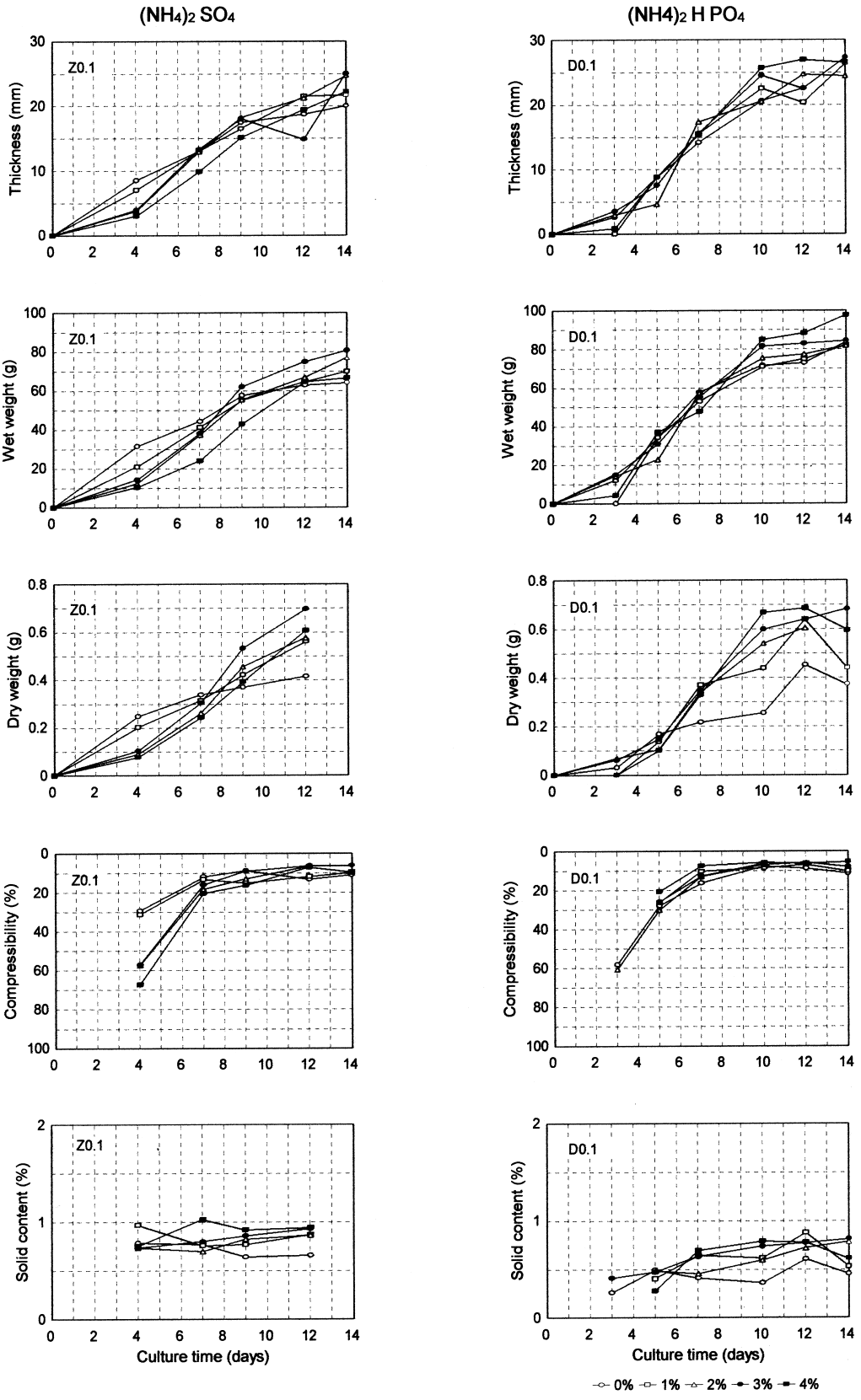


Fig. 2. Change in gel thickness, wet weight, dry weight, compressibility and solid-content during culture in coconut-water with various sugar concentrations.

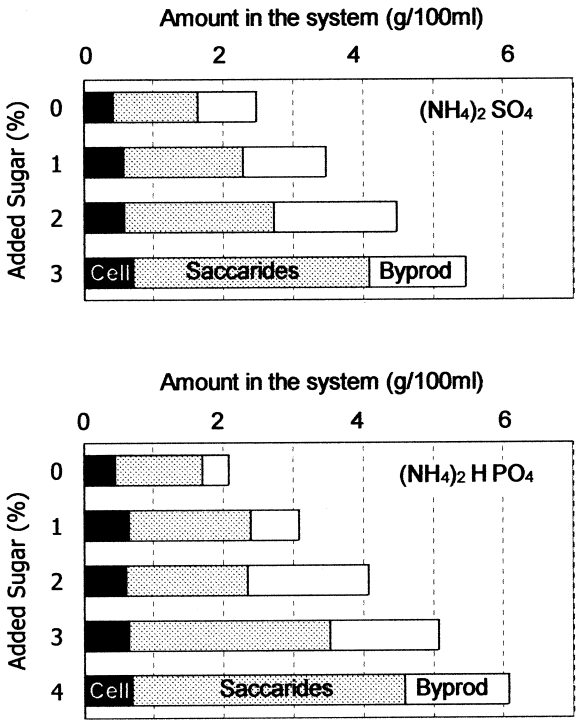


Fig. 3. Amounts of cellulose, total saccharides and byproducts (i.e. products other than cellulose), existed in the systems after 12-days culture time.

2–3 days. Of the two nitrogen-source compounds commonly of use in *nata-de-coco* industry, $(NH_4)_2HPO_4$ which also contains phosphate seemed to be slightly more effective.

Not much difference is seen between curves obtained at different sugar levels at least when this was above 1%. The amounts of cellulose, total saccharides and byproducts (i.e. the fraction of saccharides converted to something other than cellulose), existing in the systems after 12 days are shown in Fig. 3 and the results of chromatography analysis in Fig. 4. It is apparent that the added sugar was of not much use for increasing the yield of cellulose.

The changes of glucose and sucrose concentrations with culture time are plotted in Fig. 4. In common with the two series of runs with different nitrogen-source compounds, the glucose concentration did not necessarily decrease monotonically, particularly when the concentration of added sugar was high, whereas the sucrose concentration decreased monotonically towards zero. It should be noted that fructose which would have been generated by the hydrolysis of sucrose was not found.

Supplementary experiments were carried out in order to clarify the above points. In a series of runs with similar media obtained from different lot of coconut fruits, it was found that, after the addition of seed broth, the concentrations of glucose and sucrose changed very fast and reached the level of 3–4 days within a matter of 2 h. Also, some fructose was detected in the early stage, 1–2 h, when added sugar concentration was non-zero. No other saccharides but sucrose

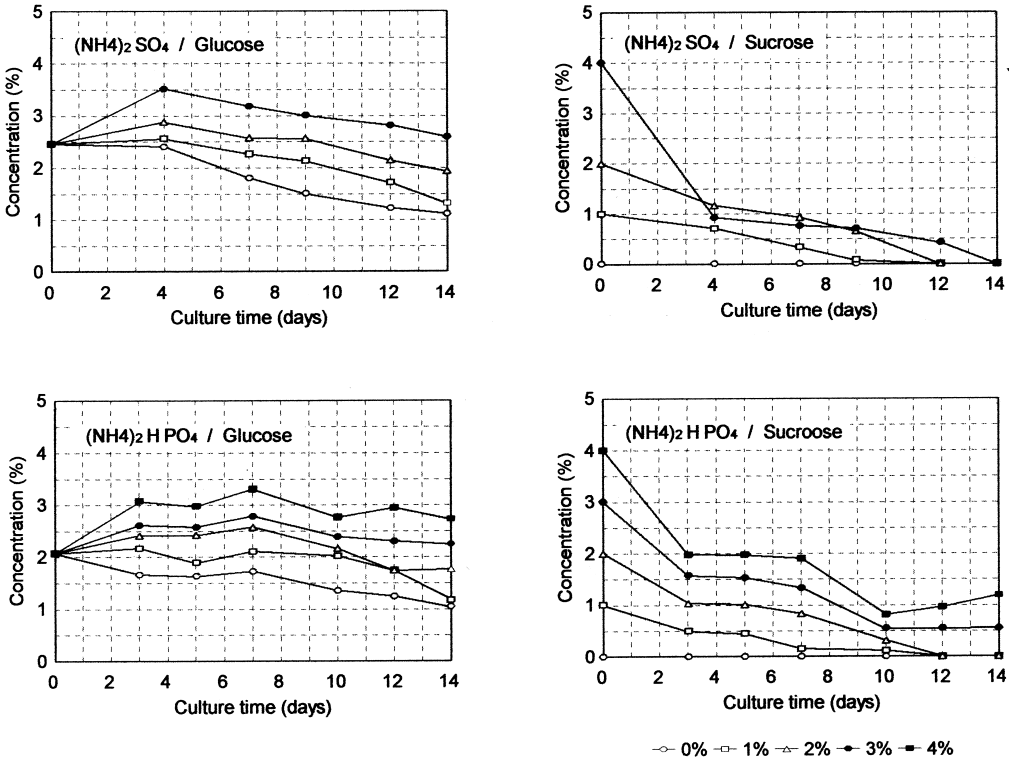


Fig. 4. Change in concentrations of glucose and saccharose during culture in coconut-water with various sugar concentrations.

was found in freshly obtained coconut-water but only glucose was detected after 3-day storage. It seems probable that enzymatic action is responsible but the details have yet to be established.

## 4. Discussion

The mechanism of gel growth is considered as follows in line with the previous explanation (Yamanaka et al., 1989; Iguchi & Yamanaka, 1997). In the initial stage, the bacteria increase their population by taking dissolved oxygen and produce a certain amount of cellulose in the entire liquid phase, as observed by the appearance of turbidity. When the dissolved oxygen is used up by ca. 3 h, bacteria existing only in the vicinity of surface can maintain their activity and produce cellulose in the form of gel. Although they may undergo cell division, the population in the surface region does not increase exponentially but should reach a certain equilibrium number, as excess bacteria diffuse into the gel interior. (Those bacteria below the surface of gel are dormant, and can be reactivated and used as the seed for new culture operation.) In fact, it was demonstrated before that the productivity of cellulose is proportional to the surface area of culture vessel and unchanged by the volume of culture medium (Masaoka et al., 1993). Thus, the main process of cellulose formation is considered to be controlled by oxygen diffusing from the atmosphere, and this is the reason why the rates of growth, represented by the slopes of curves in Fig. 1, are almost the same without depending on the concentration of saccharides. Whether oxygen pressure higher than in air accelerates the cellulose production is a different matter and has been considered elsewhere (Watanabe & Yamanaka, 1995).

As 'chemical' reactions, the system should include the hydrolysis of sucrose into glucose and fructose as well as various side reactions besides cellulose-forming reaction.

The fact that the concentration of sucrose dropped sharply to a certain level immediately after the addition of seed broth would suggest that the rate of hydrolysis was very fast but the reaction soon reached an equilibrium. In fact, the concentration of glucose is not proportional to the concentration of added sugar, and decreased slowly or stayed at a certain level, whilst the sucrose concentration decreased monotonically. Although side reactions such as those forming acetic acid, gluconic acid, etc. may not be ignored, it is probable that glucose was converted mainly to cellulose. That conversion is well known and has been verified in test cultures conducted in artificial medium without additional sugar. That no fructose was found in the system was an unexpected observation, although some was detected in the very early stages in separate runs. Since the existence of isomerising enzyme was unlikely, this saccharide would have to be consumed, no sooner than the addition of seed broth, by side reactions. An additional experiment with artificial medium containing only fructose as a carbohydrate source did not produce cellulose using the same bacterial strain, although a special species effective for fructose has been reported recently (Kojima, Seto, Tonouchi, Tsuchida & Yoshinaga, 1997). Direct consumption of sucrose by this bacterium is assumed to be impossible.

Quantitative argument on the kinetics should await a full analysis of the byproducts. As seen in Fig. 3, saccharides converted to unknown substances amounted to 30–50%, whilst the conversion to cellulose was up to ca. 20% even in favourable conditions.

Leaving aside these factors, computer simulation of the cellulose formation process, or glucose consumption, viz. in the second stage, was conducted. It was assumed that oxygen initially dissolved in the medium had been consumed and the population of bacteria increased sufficiently to be able to form a gel, and that only bacteria which lie beneath the surface and associate with oxygen diffusing from air were active. If mass transfer by convection is neglected, the change in oxygen and glucose concentrations, $C_O$ and $C_g$, respectively, in the medium at the depth, $x$ is given by

$$-\frac{\partial C_o}{\partial t} = -\frac{D_o \partial^2 C_o}{\partial x^2} + KC_oC_g \tag{1}$$

$$-\frac{\partial C_g}{\partial t} = \frac{D_g \partial^2 C_g}{\partial x^2} + KC_oC_g \tag{2}$$

where $D_o$ and $D_g$ are the diffusion coefficients of oxygen and glucose, respectively, and $K$ is an integrated rate constant of glucose consumption. In this model, the effect of the gel layer on diffusion was not taken into account, as the solid fraction was less than 1% in volume.

For the numerical solution, the equations were rewritten as follows:

$$O_{i,1} = O_0 \qquad (\text{for } i = 1, 2, \ldots) \tag{3}$$

$$O_{1,j} = 0 \qquad (\text{for } j = 2, 3, \ldots, n) \tag{4}$$

$$O_{i,j} = O_{i,j-1} - [KO_{i,j-1}G_{i,j-1} - D_o(O_{i-1,j-1} - 2O_{i,j-1} + O_{i+1,j-1})]\Delta t \tag{5}$$

$$O_{n,j} = O_{n,j-1} - [KO_{n,j-1}G_{n,j-1} - D_o(O_{n-1,j-1} - O_{n,j-1}) -]\Delta t \tag{6}$$

$$G_{1,j} = G_0 \qquad (\text{for } j = 1, 2, \ldots, n) \tag{7}$$

$$G_{1,j} = G_{1,j-1} - [KO_{1,j-1}G_{2,j-1} + D_g(-G_{1,j-1} + G_{2,j-1})]\Delta t \tag{8}$$

$$G_{i,j} = G_{i,j-1} - [KO_{i,j-1}G_{i,j-1} + D_g(G_{i-1,j-1} - 2G_{i,j-1} + G_{i+1,j-1})]\Delta t \tag{9}$$

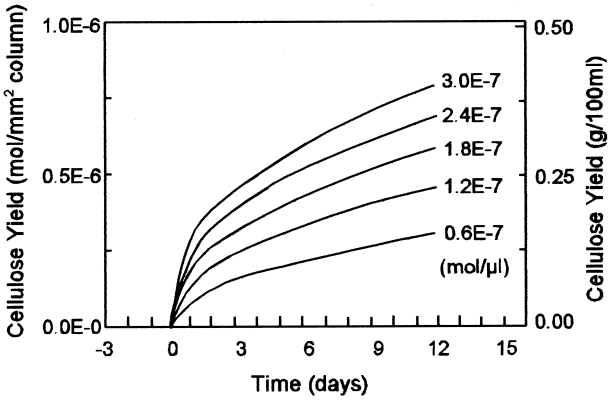


Fig. 5. A set of cellulose yield vs. time traces calculated from Eqs. (3)–(10) for $G_0 = 0.6–3.0 \times 10^{-7}$ mol/μl. Other constants and parameters assumed were: $D_o = 9.0 \times 10^{-12}$ mm$^2$/h, $D_g = 4.0 \times 10^{-12}$ mm$^2$/h, $O_0 = 1.6 \times 10^{-8}$ mol/μl and $K = 1.5 \times 10^{-8}$ μl/mol/h, and the time division, $t = 1$ h.

$$G_{n,j} = G_{n,j-1} - [KO_{n,j-1}G_{n,j-1} + D_g(G_{n-1,j-1} - G_{n,j-1})\Delta t]\Delta t. \quad (10)$$

Here, $O_{i,j}$ and $G_{i,j}$ represent $C_o$ and $C_g$ at time $i$ and depth $j$, respectively.

The practice of simulation was carried out using a spreadsheet Corel Quattro Pro 8. For the reaction system, a vessel of 30 cm$^2$ cross-sectional was considered containing a column of medium consisting of 33 layers of 1 mm$^3$ cubes was assumed in it. The time step, $\Delta t$ was set at 1 h and calculation was repeated until 288 h (12 days). $G_o$ was varied between $0.6 \times 10^{-7}$–$3.0 \times 10^{-7}$ mol/μl or ca. 1.0–5.0 g/100 ml. Other constants were floated or changed arbitrarily, starting from values obtained from chemical tables (except $K$), while the consequence was examined visually on graphical plots.

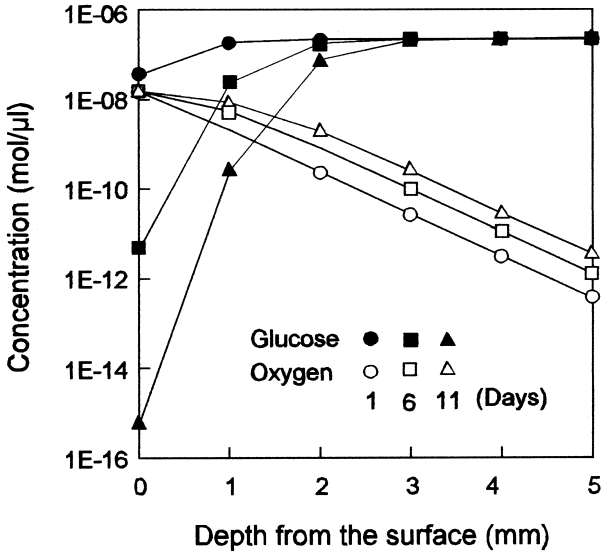


Fig. 6. Calculated concentrations of oxygen and glucose near the surface in the same condition as in Fig. 5.

Fig. 5 shows a typical time-yield traces in which the shape of curves generated appears to be reasonable. The values of diffusion coefficients, $D_o = 9.0 \times 10^{-12}$ mm$^2$/h ($2.5 \times 10^{-9}$ m$^2$/s) and $D_g = 4.0 \times 10^{-12}$ mm$^2$/h ($1.1 \times 10^{-9}$ m$^2$/s) applied are not much different from those of oxygen and organic molecules in pure water (Chem. Soc. Jpn., 1993). In fact, the diffusion coefficients had little effect on the results, even when they were changed by the factor of $10^{-2}$–$10^2$. The curves themselves are somewhat lower than those in Fig. 1 (dry weight), when the glucose concentration is referred to experimental values in Fig. 3, due possibly to ignoring the additional glucose supplied by the hydrolysis of sucrose and/or the exclusion of cellulose formed in the induction period. The increase of $K$ from the value assumed, $1.5 \times 10^{-8}$ μl/mol/h, did not improve the yield but gave irregular curves as the stepwise calculation method itself became no longer valid. In contrast to these factors, the cellulose yield tended to change drastically with the solubility of oxygen. Although $O_0 = 1.6 \times 10^{-8}$ mol/μl applied in Fig. 4 was significantly larger than the solubility of atmospheric oxygen in water, $2.58 \times 10^{-10}$ mol/μl or $5.78 \times 10^{-3}$ ml/ml at 25°C (Dean, 1992), the adoption of values of the latter level gave only a few percent yield. Whether oxygen dissolves more in glucose solution is yet to be checked experimentally.

The concentrations of glucose and oxygen near the surface of medium at three selected timings are plotted in Fig. 6. It is recognised that glucose diffuses gradually from the interior, whilst oxygen diffusing from air does not penetrate, as it is consumed by the reaction.

## 5. Conclusion

It has been confirmed that the process of cellulose formation by *Acetobacter* under static conditions is controlled by the supply of air from the medium surface and the yield depends only moderately on the concentration of saccharides. It is noteworthy that the carbohydrate contained in coconut-water after 3-day storage was only glucose and that fructose and sucrose in the sap was very unstable unless sterilised. It is plausible that cellulose is produced mainly by the consumption of glucose at least with the particular bacterium employed in this study, although full analytical data of byproducts are needed to confirm this.

For the manufacturing of *nata-de-coco*, it is useful to know that 1% sugar is more or less sufficient to be added, whereas two or more percent is currently applied in industry, and a widely adopted recipe for the preparation of synthetic culture medium (Schramm & Hestrin, 1954) includes 5% sugar among other ingredients.

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## References

Borzani, W., & de Souza, S. J. (1995). Kinetics of the bacterial production of cellulosic pellicles on non-agitated liquid media. *Biotechnol. Lett.*, *17*, 1271–1272.

Brown, A. J. (1886). On an acetic ferment which forms cellulose. *J. Chem. Soc*., *49*, 172–186.

Brown, A. J. (1886). The chemical action of pure cultivation of Bacterium aceti. *J. Chem. Soc.*, *49*, 432–439.

Brown, A. M. (1962). The mechanism of cellulose biogenesis by Acetobactor Acetigenum, *J. Polym. Sci.*, *59*, 1555–1569.

Chemical Society of Japan (1993). 4. *Chemical Handbook*, II. Maruzen.

Dean, J. A. (1992). Lange's Handbook of Chemistry. 14. New York: McGraw-Hill.

Iguchi, M., Yamanaka, S., Watanabe, K., Nishi, Y. & Uryu, M., 1991. Preparation of high-strengthmaterials from bacterial cellulose. In: Lemstra, P.J. & Kleintjens, L.A. (Eds.), *Integr. Fundam. Polym. Sci. Technol. 5*, [*Proc. Int. Meet. Polym. Sci. Technol, Rolduc Polym. Meet. 5*], London, 1990, pp. 44–50, 371–379.

Iguchi, M., & Yamanaka, S. (1997). Industrial use of bacterial cellulose—a review. *Proc. Int. Workshop Green Polym. Bandung-Bogor*, 47–54.

Iguchi, M., Mitsuhashi, S., Ichimura, K., Nishi, Y., Uryu, M., Yamanaka, S. & Watanabe, K., 1988. US Patent 4,742,164.

Kojima, Y., Seto, A., Tonouchi, K., Tsuchida, T., & Yoshinaga, F. (1997). High rate production in static culture of bacterialcellulose from sucrose by a newly isolated Acetobacter strain. *Biosci. Biotechnol. Biochem.*, *61*, 1585–1586.

Marx-Figini, M., & Pion, B. G. (1974). Kinetic investigations on biosynthesis of cellulose by Acetobactor xylinum. *Biochimica Biophysica Acta*, *338*, 382.

Marx-Figini, M., & Pion, B. G. (1976). Kinetic investigation on biosynthesis of bacterial cellulose. 2. Distribution of the degree of polymerization for different numbers of bacterial generation. *Makromol. Chem.*, *177*, 1013.

Masaoka, S., Ohe, T., & Sakota, K. (1993). Production of cellulose from glucose by Acetobacter xylinum. *Ferment. Bioengng*, *75*, 18–22.

Nishi, Y., Uryu, M., Yamanaka, S., Watanabe, K., Kitamura, K., Iguchi, M., & Mitsuhashi, S. (1990). The structure and mechanical properties of sheets prepared from bacterial cellulose. Part 2: Improvement of the mechanical properties of sheets and their applicability to diaphragms of electro-acoustic transducers. *J. Mater. Sci.*, *25*, 2997–3001.

Ring, G. J. F. (1982). A study of the polymerization kinetics of bacterial cellulose through gel-permeation chromatography. In R. M. Brown (Ed.), *Cellulose and Other Natural Polymer Systems*, (pp. 299–325). New York: Plenum.

Schramm, M., & Hestrin, S. (1954). Factors affecting production of cellulose at air/liquid interface of a culture of Acetobactor xylinum. *J. Gen. Microbiol.*, *11*, 123–129.

Watanabe, K., & Yamanaka, S. (1995). Effects of oxygen tension in the gaseous phase on production and physical properties of bacterial cellulose formed under static culture conditions. *Biosci. Biochem.*, *59*, 65–68.

Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., Nishi, Y., & Uryu, M. (1989). The structure and mechanical properties of sheets prepared from bacterial cellulose. *J. Mater. Sci.*, *24*, 3141–3145.

Yoshinaga, F., Tonouchi, K., & Watanabe, K. (1997). Research progress in the production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material. *Biosci. Biotechnol. Biochem.*, *61*, 219–224.