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Location and Limitation of Cellulose Production by *Acetobacter xylinum* Established from Oxygen Profiles

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The static fermentation of coconut water sucrose by *Acetobacter xylinum* was carried out at initial pH's of 3.0, 4.0, 5.0 or 6.0. Cellulose was produced at the surface, and its production was most favourable at pH's 4.0 and 5.0. These pH values also allowed for optimal bacterial growth. Oxygen concentration profiles were measured with microelectrodes at different cultivation stages, and steep profiles were obtained with penetration depths between 50 and 100 μm. A substrate penetration depth analysis confirmed the hypothesis that the first stage of the fermentation is entirely oxygen controlled. Diffusion calculations showed, however, that at a later stage sucrose becomes a limiting substrate also, which was confirmed by the decrease in cellulose production rate over time. The effective diffusion coefficient of oxygen in deactivated cellulose pellicles was measured with microelectrodes, and a value of 1.4×10^{-9} m^2/s was obtained under all investigated conditions. The oxygen flux was 5.9×10^{-6} mol/m$^2 \cdot$s, while a significantly higher value of 9.1×10^{-6} mol/m$^2 \cdot$s was obtained at pH 4.0.

[Key words: oxygen profile, diffusion, microelectrode, *Acetobacter xylinum*, nata de coco]

Microbial cellulose, in the Philippines known as *nata de coco*, is used in sweetened form as dessert, as an ingredient in food products and cosmetics, and as an additive in the manufacture of cloth, high grade papers, and membrane materials (1, 2). It is produced at the surface of coconut water and other suitable media by a gram-negative rod-shaped bacterium, *Acetobacter xylinum* (3). The fermentation is carried out in static cultures at 30°C in acidic pH from 3.5 to 7.0. As the organism is obligate aerobic, it is thought that cellulose is produced at the air/liquid interface, where the cells can obtain oxygen (4). In static cultures, substrates have to be transported entirely by diffusion, and as carbon sources are generally available, the oxygen supply is considered as the limiting factor for growth and cellulose production. Moreover, diffusion rates will be reduced gradually due to the formation of cellulose at the surface. To date, there is no information available concerning the oxygen concentration in the medium or the changes in the local oxygen concentration during fermentation. However, it is quite likely that substantial concentration profiles will develop.

In this study, we investigated the influence of the initial pH in the relevant range of 3.0–6.0 on the rate of cellulose production, the development of oxygen concentration gradients, and the effective diffusion coefficient D_e of oxygen in the produced pellicle. Microelectrodes with tip diameters in the μm range have been employed successfully in the field of biotechnology to measure the amount of dissolved oxygen for the past two decades (5–8). A high spatial resolution can be achieved, enabling the measurement of local concentration gradients inside biofilms without disturbing them. To determine the oxygen diffusion coefficients, we applied the transient state method as described by Cronenberg and Van den Heuvel (9). Microelectrodes were positioned at a fixed place in-

side the deactivated biofilm pellicle, followed by a stepwise concentration change in the bulk of the liquid, and the subsequent response was monitored as a function of time.

MATERIALS AND METHODS

General All reagents and chemicals used were of analytical grade, unless stated otherwise.

Organism The *Acetobacter xylinum* strain was isolated from *nata de coco* and was supplied by Kasetsart University, Bangkok, Thailand.

Experimental fermentations For the purpose of oxygen concentration measurements, fermentations were carried out in test tubes (ϕ15 mm) containing 9.5 ml of sterilized and pH-adjusted coconut water sucrose (CWS) medium. Each tube was inoculated with 0.5 ml of a preculture grown at 30°C for 72–96 h in a rotary shaker, giving an initial cell density of log CFU (colony forming units)/g=7.5. For each pH value, 12 replicate fermentation tubes were inoculated and incubated at 30°C under static conditions.

Media The CWS medium consisted of 20.0 g sucrose, 5.0 g (NH$_4$)$_3$PO$_4$, and 5.0 g glacial acetic acid dissolved in 1.0 *l* of coconut water. Coconut water was obtained from coconuts purchased locally (Wageningen). The initial pH was adjusted to 3.00, 4.00, 5.00 or 6.00 with 0.1 M H$_3$PO$_4$ or sterilized 0.5 M NaOH. The composition of the peptone saline solution (10) was 1.0 g of bacto-peptone, and 8.5 g of sodium chloride in 1.0 *l* of distilled water.

The composition of the modified Schramm, Gromet and Hestrin (SGH) plating medium (11) was 20.0 g of glucose, 5.0 g of yeast extract, 5.0 g of bacto-peptone, 1.0 g of K$_2$HPO$_4$, 10.0 g of agar, and 5.0 g of glacial acetic acid in 1.0 *l* of distilled water. All media were autoclaved for 20 min at 121°C. Glacial acetic acid was

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added aseptically after the media had cooled below 50°C.

Oxygen microelectrodes and profiles   Combined oxygen microsensors with a tip diameter of 10 μm were prepared according to the method of Revsbech and Ward (12). The electrode was mounted vertically on a motor-driven micromanipulator and placed inside a Faraday cage to reduce electromagnetic interference. The electrical current was measured with an amperometer (Keithley 617, Cleveland, USA). The test tubes were removed from the incubator and immersed in a 30°C water bath. Due to the roughness of the surface of the pellicle on a microscopic scale, a precise visual determination of the interface was impossible. Hence, the electrode was lowered at steps of 1 μm until a decrease in the signal was observed, and this point was considered to be the film/air interface.

From days 1 to 7 after inoculation, two different tubes from every set of initial pH values were randomly selected for the oxygen profile measurements. From day 9 onwards, the same set of two tubes was used, and profiles were measured in the center of the pellicle and at a distance of 4 mm from the edge of the tube.

Oxygen diffusion coefficient   To determine the effective diffusion coefficient (D_e), a transient state measurement was carried out in pellicles grown at different initial pH values. At the end of the fermentation the microbial activity was inhibited by adding a solution of sodium azide (final concentration 0.2%). The tubes were exposed to air for 1 week, allowing the contents to be fully saturated with air. Due to the 'elasticity' of the pellicle material, which induces a compressed diffusion barrier in front of the electrode tip, the output of the electrode was severely reduced when the penetration depth exceeded 300 μm. Similar problems have been described in the literature regarding several immobilization materials, and for details the reader is referred to Müller et al. (13). Therefore, a special procedure was required, viz., the tip of the microelectrode was driven first to a depth of approximately 1500 μm, and subsequently withdrawn to the desired depth of 1000 μm. In general, this was sufficient to regain the calibration signal; however, in some cases this procedure had to be repeated several times. Unfortunately, a stepwise increase in the concentration by means of gaseous oxygen decreased the pellicle thickness during the measurement due to the evaporation of water. Therefore, after a stabilization period of 30 min for the electrode a stepwise increase in oxygen concentration from 0.24 to 1.14 mol/m³ was applied by adding 2 ml of water saturated with pure oxygen on the top of the pellicle. During the measurement, pure oxygen was continuously bubbled through this liquid layer to keep it oxygen-saturated, and to minimize the stagnant liquid boundary layer. The resulting changes in the output of the microelectrode were monitored continuously for about 3 h by means of a recorder. The effective diffusion coefficient (D_e) was obtained by fitting the time-dependent oxygen concentration C to the theoretical response for a flat slab (14):

$$\frac{C-C_0}{C_s-C_0}=1-\frac{4}{\pi}\cdot\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\cdot\cos$$

$$\left[(2n+1)\frac{\pi r}{2L}\right]\cdot\exp\left\{-\left[(2n+1)\frac{\pi}{2L}\right]^2 D_e\cdot t\right\} \qquad (1)$$

where C_s is the oxygen concentration in the bulk liquid (mol/m³), C_0 the initial oxygen concentration, L the total depth of the slab $(5\times10^{-2}\,m)$, r the position of the electrode from the bottom of the slab $(4.9\times10^{-2}\,m)$, D_e the effective diffusion coefficient (m²/s), and t the time (s). The signal was corrected for the drift of the electrode (less than 2%/h), and all measurements were carried out in duplicate. To simplify the calculations it was assumed that the mass transfer resistance due to the stagnant liquid boundary layer at the top of the pellicle was negligible, and that the medium exhibited the same diffusion coefficient as the cellulose pellicle.

Oxygen fluxes   The oxygen flux J through the interface was calculated according to Fick's first law of diffusion:

$$J=-D_e\,(dC/dx) \qquad (2)$$

where J is the flux of oxygen (mol/m²·s), and dC/dx the local oxygen concentration gradient. C equals 0.24 mol/m³ at 30°C.

Moisture content   The cellulose pellicle was lifted with tweezers, weighed and subsequently dried in an oven for 16 h at 80°C. The moisture content (% w/w) was calculated based on the weight loss of the pellicle.

Total viable counts   The pellicle was cut into small pieces with sterile scalpels, and placed in a sterile plastic bag. Then, a peptone saline solution 9-fold the weight of the cut pellicles was added, and the contents were pressed manually for 2 min to obtain a tenfold diluted suspension. The number of viable cells (TVC) expressed as log CFU/g was determined using serial decimal dilutions on agar plates with the SGH medium. Colonies were counted after 4 d of incubation at 30°C.

RESULTS

Pellicle development   The effect of the initial pH in the range 3.0 to 6.0 on the cellulose production rate of *Acetobacter xylinum* was determined. Figure 1 shows that cellulose formation is the most favourable at an initial pH of 5.0, with pH 4.0 as the next best. The deviation in the final pellicle thickness was ±5%. At pH 6.0 the initial formation was retarded, but from day 3 onwards the same cellulose synthesis rate was observed. Cellulose formation rates gradually decreased over the course of time, and beyond day 11 the rate was reduced to about 25%. The changes in total viable counts
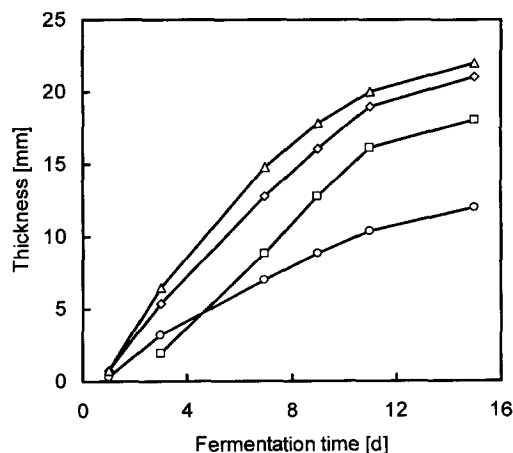
FIG. 1.   Pellicle thickness during the course of fermentation at initial pH values of 3.0 (O), 4.0 (◇), 5.0 (△), and 6.0 (□).

(TVC), pH and moisture content are summarized in Table 1. The concentration of viable cells decreased at all investigated pH values. Among the tested pH values, the highest cell counts on days 3 and 7 were obtained at pH 4.0. On the other hand, the highest cell counts on day 9 and beyond were obtained at pH's 4.0 and 5.0. The pH value of the medium remained within 0.2 units of the initial pH, except for pH 6.0, which gradually decreased to 5.37. The moisture content was the same for all pellicles, irrespective of the initial pH value and the cultivation time.

Oxygen profiles   Figures 2a–d show the development of the oxygen concentration gradients in the course of the fermentation at initial pH values of 3.0, 4.0, 5.0, and 6.0, respectively. After one day, the steepest profiles were observed at pH 4.0, reducing the oxygen penetration depth to less than 200 μm. For the same period, a penetration depth of 400–500 μm was observed at pH's 3.0 and 5.0, while at pH 6.0, air saturation still amounted to 30% at a depth of 2 mm (data not shown). On day 3, the reaction zone was limited to 100 μm or less, except at pH 6.0. On day 7, all of the oxygen profiles were less than 100 μm, while those at pH 4.0 were reduced even further to 50 μm. These are typical values often ob-

served for biological systems (5, 6, 8, 15). Beyond day 7 no significant changes in the slopes of the oxygen profiles were observed.

Diffusion coefficients   After deactivation of the microorganisms with sodium azide, oxygen gradients were no longer observed. Reproducible responses were obtained, and a characteristic response to a stepwise increase in the oxygen concentration is shown in Fig. 3. For this specific pellicle (pH 4.0), a good fit was obtained with a D_e value of 1.4×10^{-9} m^2/s. No significant differences were found between diffusion coefficients in cellulose produced at different initial pH values. The average D_e for oxygen was 1.4 (± 0.2) $\times 10^{-9}$ m^2/s.

Oxygen fluxes   Oxygen fluxes were calculated from the linear part of the slopes of the profiles (Fig. 2) and the diffusion coefficient D_e, according to Fick's first law of diffusion. The average fluxes were calculated from 2–4 measurements and are presented in Table 1. Oxygen fluxes did not change after day 3, except at pH 6.0 where a constant flux was reached from day 7 onwards. The average oxygen flux between day 3 and 15 was 9.1 (± 0.8) $\times 10^{-6}$ mol/m$^2 \cdot$s at pH 4.0, while at the other pH values, a much lower average value of 5.9 (± 1.1) $\times 10^{-6}$ mol/m$^2 \cdot$s was obtained.

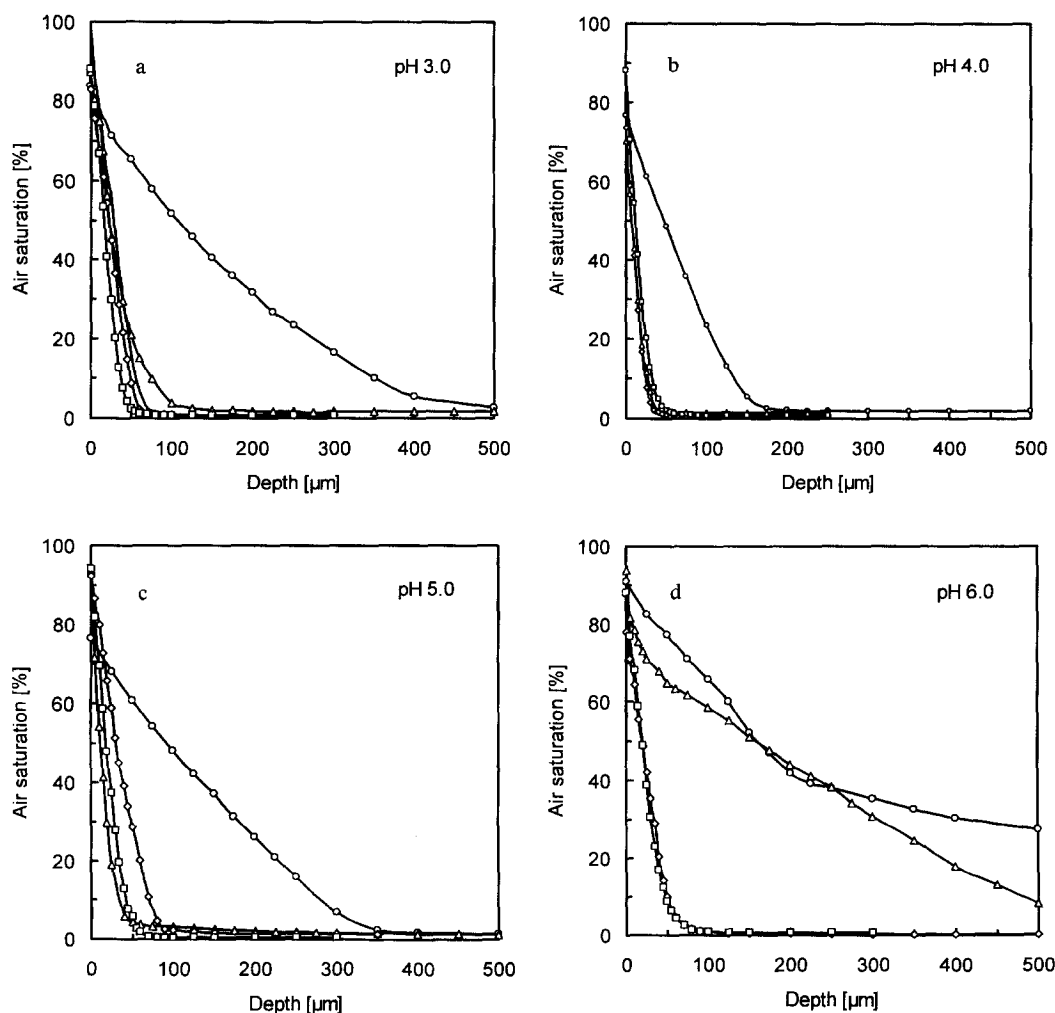FIG. 2.   (a–d) Typical oxygen profiles at an initial pH of 3.0, 4.0, 5.0, and 6.0 at day 1 ($\bigcirc$), 3 ($\triangle$), 7 ($\diamond$), and 15 ($\square$) of fermentation.

TABLE 1. Changes in oxygen flux, total viable count (TVC),
moisture content, and pH during the course of
fermentation at different initial pH values

Initial (pH)	Parameter	Fermentation (d)					
		1[a]	3	7	9	11	15
3.00	Flux (10^{-6} mol/m$^2\cdot$s)	1.8	5.8	5.5	4.2	8.5	6.8
	TVC (log CFU/g)	nd	6.8	6.1	nd	nd	2.5
	Moisture (% w/w)	nd	88.6	90.8	90.9	90.7	90.8
	pH	nd	3.09	3.01	3.04	3.00	2.96
4.00	Flux (10^{-6} mol/m$^2\cdot$s)	4.0	7.8	9.3	9.1	9.2	9.9
	TVC (log CFU/g)	nd	8.1	7.3	6.0	5.9	5.7
	Moisture (% w/w)	nd	90.2	88.9	91.1	91.2	91.1
	pH	nd	3.92	3.88	3.93	3.83	3.82
5.00	Flux (10^{-6} mol/m$^2\cdot$s)	1.3	7.2	4.7	6.0	5.2	6.4
	TVC (log CFU/g)	nd	6.5	6.3	6.3	5.2	5.7
	Moisture (% w/w)	nd	90.2	90.4	90.3	90.4	90.4
	pH	nd	4.80	4.80	4.91	4.83	4.85
6.00	Flux (10^{-6} mol/m$^2\cdot$s)	1.3	0.67	5.1	5.4	6.2	5.2
	TVC (log CFU/g)	nd	nd	nd	5.3	4.5	4.5
	Moisture (% w/w)	nd	nd	nd	90.1	90.5	90.2
	pH	nd	5.64	5.52	5.54	5.44	5.37

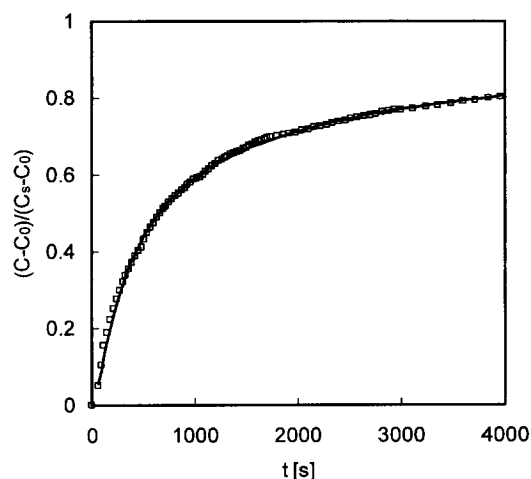[a] Flux calculated with D of oxygen in water.
nd, Not determined.

FIG. 3. Typical response to a stepwise increase in oxygen concentration in a cellulose pellicle (initial pH 4.0) measured with a microelectrode. The solid line indicates the theoretical response at a depth of 1000 μm and with a diffusion coefficient D_e of 1.4×10^{-9} m^2/s.

DISCUSSION

The highest cellulose formation rate at initial pH values of 4.0 and 5.0 is in good agreement with results of earlier studies (16–18). These pH values also allowed for optimal bacterial growth, and coincide well with pH 4.5 as the optimum reported by Embuscado et al. (17), particularly with regard to strain variety (2). The overall cellular densities were low, but in the same order as those obtained by Embuscado et al. (17), viz., 5–7 log CFU/g. However, if we assume that most of the viable cells are concentrated in the oxygen-penetrated zone for pH 4.0 on day 7, log CFU/g would be 7.3+log 260=9.8, since the thickness of the pellicle was 13 mm (Fig. 1), and the upper active layer was 50 μm (Fig. 2b). According to Hestrin and Schramm (19), the concentration of freeze-dried A. xylinum cells is approximately 10^{12} cells/g, corresponding to 11 log CFU/g for hydrated cells, assuming 10% dry weight. Thus, if our hypothesis is correct, the upper layer consists of about 10% of cells. Hestrin and Schramm (19) also determined the oxygen uptake rate of pure cells (10^{17} cells/m^3) of A. xylinum to be 1.2 mol/m$^3\cdot$s. The oxygen consumption rate R (mol/m$^3\cdot$s) can be calculated from the oxygen flux $J=R\cdot\delta$, in which δ (m) is the depth of the reaction zone. For day 7 at pH 4.0 (Table 1), with $J=9.3 \times 10^{-6}$ mol/ m$^2\cdot$s and $\delta=50\,\mu$m, a value of 0.19 mol/m$^3\cdot$s is obtained, which is about 15% of the activity of pure cells. Although a rough estimation, this value corresponds well with the previously estimated concentration of 10% of cells.

On days 3 and 7 of the fermentation at pH 4.0, the steepest oxygen profiles corresponded well with the highest cell concentrations (TVC). Interestingly, from day 9 onwards, the oxygen flux at pH 4.0 was 50% higher than that at pH 5.0, although the cell counts were virtually the same for cultures at pH's 4.0 and 5.0. Notwithstanding the higher oxygen flux at pH 4.0, the same amount of cellulose was produced per unit of time at pH 5.0. Furthermore, at pH 3.0 oxygen fluxes equalled those of pH 5.0, while the cellulose production rate was

only 50%. Similar results were obtained by Hestrin and Schramm (19): at pH 3.0 their cellulose synthesis rate was only 20% compared to that at pH 5.0, while the corresponding oxygen uptake rate was 75%. The continuous decrease in the number of viable cells in the course of fermentation, and the decrease in cellulose formation rate beyond day 11 did not affect the oxygen concentration profiles, i.e., oxygen uptake rates. These results indicate that there is no direct relationship between cellulose formation and oxygen demand, and consequently other oxygen-consuming processes have to play a role. A. xylinum is known to oxidize glucose at high concentrations to gluconic acid with a concomitant pH decrease (16, 17, 20, 21). Unfortunately, the high-buffer capacity of the medium in the lower pH range would have prohibited this decrease, and except at pH 6.0, no significant pH decrease was observed. Another explanation might be that at less favourable lower pH values, more oxygen is used for cell maintenance.

Borzani and de Souza (22) found by means of tracer experiments that the formation of cellulose occurs at the film/air interface. Masaoka et al. (16) came to the same conclusion, yet no fundamental mechanism was proposed by these authors. Our experiments, however, determined a 50–100 μm active upper zone in which cellulose is formed exclusively. It should be emphasized that such steep oxygen profiles are typical of various biological systems (5, 6, 8, 15). Due to oxygen limitation, which occurs in the early stage of fermentation, cell growth and cellulose production only occur in this narrow zone at the air/medium interface. The cellulose pellicle floats on the liquid, and the produced cellulose is pushed down in the course of time, while new cellulose layers are constantly built on top of the mature cellulose. Three factors are involved in this phenomenon: (i) the transport distance of sucrose to the reaction zone at the top is much greater than that for oxygen; (ii) the diffusion coefficient of sucrose is almost five times smaller than that of oxygen in water, i.e., 6.2×10^{-10} and 2.8×10^{-9} m^2/s (23, 24), respectively; and (iii) the maximum oxygen concentration in the reaction zone is much less than the sucrose concentration in the broth (0.24 vs. >58 mol/m^3).
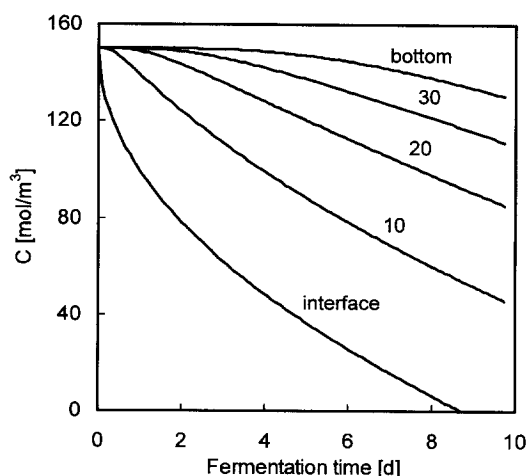
FIG. 4. Calculated sucrose concentration with respect to time at different depths, *i.e.*, the reaction zone/medium interface, at depths of 10, 20, and 30 mm, and the bottom.

If the hypothesis that cellulose is only formed in the reaction zone is correct, then the penetration depth of sucrose from below should be significantly larger than that of oxygen from above.

Assuming a zero-order kinetics, the penetration depth (δ) can be estimated by (25):

$$\delta = \sqrt{\frac{2D_e C_i}{R}} \qquad (3)$$

where C_i is the sucrose concentration at the interface (mol/m^3). The sucrose consumption rate R can be estimated from a simplified metabolic reaction:

$$\text{Sucrose} + 12O_2 \rightarrow 12CO_2 + 12H_2O \qquad (4)$$

$$\text{Sucrose} \rightarrow \text{Cellulose} \qquad (5)$$
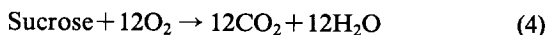
Hence, sucrose is consumed for two purposes. From Fig. 1 it can be seen that 2 mm of pellicle is formed per day between days 3 and 7 at pH 4.0. With a tube diameter of 15 mm, an assumed density of 10^3 kg/m^3, and a dry matter content of 10% (Table 1) this translates into a maximum cellulose production rate of 4.0×10^{-10} kg dw/s on day 7. With a molecular weight of 342 for sucrose, and a reaction zone 50 μm thick where both oxygen and sucrose are consumed (Fig. 2b), the estimated sucrose uptake rate for pellicle formation is 0.13 mol/m$^3 \cdot$s. The consumption rate for oxygen at day 7 was calculated earlier to be 0.19 mol/m$^3 \cdot$s. The concomitant sucrose consumption rate for growth and maintenance can be derived from Eq. 4 and is determined to be 0.016 mol/m$^3 \cdot$s. Thus, the total sucrose consumption rate adds up to 0.15 mol/m$^3 \cdot$s. Assuming that C_i is equal to the initial bulk concentration, a condition valid only in the early stage of fermentation, Eq. 3 yields a maximal penetration depth of 500 μm. Thus, the penetration depth for sucrose is 10 times larger than that for oxygen, indicating that for the early stage of fermentation, oxygen is indeed the limiting substrate.

In a later stage, sucrose will become a limiting substrate also, due to the large transport distance. The static medium can be considered to be a plane sheet with a uniform initial concentration C_0, one impermeable bottom surface and a constant sucrose flux from the reaction zone/medium interface (14). Figure 4 shows the calculated sucrose concentration with respect to time at different depths, *i.e.*, the reaction zone/medium interface, at depths of 10, 20, and 30 mm, and the bottom. The initial sucrose concentration was assumed to be 150 mol/m^3, since coconut water contains approximately 30 g/*l* saccharides (26), while the reaction rate was assumed to be 50% of the previously estimated value because part of the dry weight is composed of salts and non reacted sugars. Since only a small part of the sheet consists of cellulose, the diffusion coefficient for water was used for simplicity. According to these calculations, the sucrose concentration at the interface will approach zero, long before its concentration in the medium is exhausted. In actual conditions, however, the reaction rate and thus the flux will decrease when the sucrose concentration at the interface nears the saturation constant K_s. These calculations are confirmed by the gradual decrease in the cellulose production rate over time. From Fig. 4 it is also clear that the macroscopic determination of the sucrose concentration in the bulk liquid at best will only yield an average concentration, and will not yield any information at all concerning the relevant concentration at the interface.

The measured diffusion coefficient for oxygen in the cellulose pellicle is in good accordance with those in several gel support materials, *e.g.*, D_e values of 1.5×10^{-9} to 2.1×10^{-9} m^2/s were obtained for 0.5–5% w/v κ-carrageenan, alginate and gellan gum, respectively (27, 28). As the moisture content and diffusion coefficients were very similar for pellicles grown at different pH values, it seems reasonable to conclude that the pH has no influence on the physical properties of cellulose produced by *A. xylinum*.

Conclusion   The mechanism underlying cellulose formation was established; the oxygen profiles unequivocally demonstrate that the fermentation of coconut water sucrose by *A. xylinum* occurs at the film/air interface zone of 50–100 μm. A penetration depth analysis showed that during the early part of fermentation, oxygen was indeed the limiting substrate. However, calculations showed that in a later stage, sucrose also becomes a limiting substrate, and this might explain the gradual decrease in the cellulose production rate.

The effective diffusion coefficient (D_e) for oxygen was 1.4×10^{-9} m^2/s, which is about 50% of that for water. The diffusion coefficient and moisture content of the cellulose pellicles were not influenced by the pH. The pH for optimal cellulose production and bacterial growth is between 4.0 and 5.0, while the highest oxygen uptake rate occurs at pH 4.0.

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