0273-2289/97/63–65—0327$11.00

Acetobacter Cellulosic Biofilms Search for New Modulators of Cellulogenesis and Native Membrane Treatments

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ABSTRACT

Since natural substances like pseudoxanthins exert a positive effect on the cellulogenic ability of *Acetobacter xylinum* when producing cellulosic pellicles suitable for skin burn therapy, new defined and complex modulators were sought. Ca^{2+} and Mg^{2+} (4 m*M*) were strongly stimulatory. Na^{+} had no effect and K^{+} was inhibitory. Ammonium dihydrogen phosphate (0.12 g/L) ensured the same nitrogen supply as the same concentration of yeast extract as measured by cellomembrane dry wt./yield albeit higher yeast extract supplies produced thicker membranes. Corn steep liquor (CSL) was also progressively beneficial from 0.125 to 0.5 mL/L, and this yield could be further improved by the combination of CSL with a tea infusion (source of caffeine). Uridine (precursor for UDP-Glc, sugar donor in cellulose biosynthesis), guanine, guanosine, and its butirylated derivatives (precursors for the positive modulator of cellulose synthetase, di-cGMP) resulted in only moderate stimulation. Sodium phytate and betaine were also slightly stimulatory. The fibrilar product from

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a new *Acetobacter* isolate (Ax-M) was characterized as cellulose by comparison with the solid-state ^{13}C-NMR of algal cellulose. Its X-ray diffractogram was a confirmatory analysis. After incorporation of tamarind xyloglucan to previously air-dried cellulosic pellicles, diffractometry displayed only slight differences. Mercerized (5*M* NaOH) fresh cellulosic biofilms underwent drastic size reduction (3.5-fold), turning compact nut still flexible if maintained wet.

Index Entries: *Acetobacter*; cellulosic biofilms; cellulogenesis modulation; bacterial cellulose.

INTRODUCTION

Bacterial cellulose has well-established applications as a (bio)technological tool. Examples are: artificial skin in the therapy of burns and other dermic injuries *(1)*, high-fidelity acoustic speakers *(2)*, high-quality paper *(3)*, ultrafiltration membranes, cover membrane for glucose biosensors, culture substrate for mammalian cells, binder/thickener for paint, ink, and adhesives, and finally, diet and dessert foods *(4)*. These applications are owing primarily to such properties as large surface area, high water-holding capacity, moldability, and strong shear resistance.

The scientific and technological research carried out on bacterial cellulogenesis has yielded some remarkable findings. A novel cyclic dinucleotide c(GMP)$_2$ was identified and characterized as the cellulose synthetase positive modulator *(5)*.

The positive metabolic role of pseudoxanthines from tea infusions (caffeine and theophylline), as potent stimulators of *Acetobacter xylinum* cellulosic biofilms by targeting the c(GMP)$_2$-destroying phosphodiesterases, has also been elucidated *(6)*.

Mixed fibers and composites from bacterial cellulose were first developed at the University of Texas at Austin *(7)*. A doubled yield of *A. pasteurianus* cellulose was achieved by including an oxygen-permeable membrane in the fermentation vessel *(4)*. New wild-type and mutant hyperproducing strains and a subsp. (*sucrofermentans*) of *A. xylinum* were recently described *(8,9)*. Culture media optimization for the production of bacterial cellulose was surprisingly achieved by adding endoglucanase, a cellulose depolymerizing enzyme *(10)*.

We routinely use static cultures of *Acetobacter* and collect the floating and progressively thickened cellulosic membrane at the medium surface line. The Rensseler Polytechnic Institute (Troy, NY) uses a rotating disk bioreactor *(11)*. Scaling up of bacterial cellulose production for several commercial applications was accomplished by a collaborative project between Weyerhaeuser Co. (Tacoma, WA) and Cetus Corporation, thus leading to a multipatent-protected product, Cellulon™ (Folster, H. G., lecture 28, 17th Symposium on Biotechnology for Fuels and Chemicals, Vail, CO, May 1995).

Following our previous contributions on the subject of bacterial cellulose biosynthesis *(1,6)*, a comparative study is now being undertaken of the responses of a new isolate (Ax-M) and a collection reference strain (Ax-G) of *A. xylinum* to several metabolic effectors of cellulogenesis.

MATERIALS AND METHODS

Strains—Origin and Maintenance

Ax-M, under PCR-based genetic characterization, is an isolate of *Acetobacter* sp. (presumably *xylinum*, collected by M A. Fontana) from a fermented coffee. Ax-G is a reference strain of *Acetobacter aceti* subsp. *xylinum* (DSM 2325; GBF, Braunschweig, Germany) received as a gift by M. Baron. Stock cultures were maintained as lyophilized mixture of cells and skim milk.

Routine Liquid Medium for Nonagitated Cultures

The nonstimulated medium (BM^-) contained, per liter: glucose (40 g), yeast extract (0.1–0.2 g), ammonium dihydrogen phosphate (0.1 g), and 5 mL ethanol. Except in the experiments using *Camellia sinensis* (tea) infusions, caffeine, and theophylline (2 mg/L) were included as stimulators (BM^+). All other additions are detailed in the text (Results and Discussion). Corn steep liquor (CSL) was provided by Refinaçoes de Milho Brasil (Balsa Nova, PR, Brazil) as a concentrate with ca. 40 g% solids. Its particulate matter was removed before use by centrifugation at 10 krpm (12,300 g). Liquid cultures were in a final volume of 100 mL in glass vessels of 250 mL with a slight reduction of the diameter from the top to bottom in order to avoid accidental submersion of the growing and floating cellulosic membrane. Fermentation vessels were covered with a double layer of micropierced napkin sheets in order to allow free air/gas exchange. Nystatin and benomyl (5 mg/L each) were added to all culture media in order to avoid yeast and/or mold contaminations. A suspension of bacterial cells (0.15–0.20 OD units at 650 nm) was used at 1/20 vol inoculum. Growth was at 28°C for 5 d without agitation. Cellulose pellicles, prior to gravimetric quantitation, were exhaustively washed with distilled water to remove all water-soluble contaminants and then lyophillized.

Physical Analyses

Diffractometry and electron microscopy were carried out as previously described *(1)*. For the diffractogram and solid-state ^{13}C-nuclear magnetic resonance (NMR) the cellulosic membranes were pretreated by extensive washing with 1% sodium dodecyl sulfate (SDS). Tamarind seed xyloglucan (Ts-XG) was obtained through five boiling cycles (microwave oven) of an aqueous suspension of seed endosperm, followed by centrifugation at 10

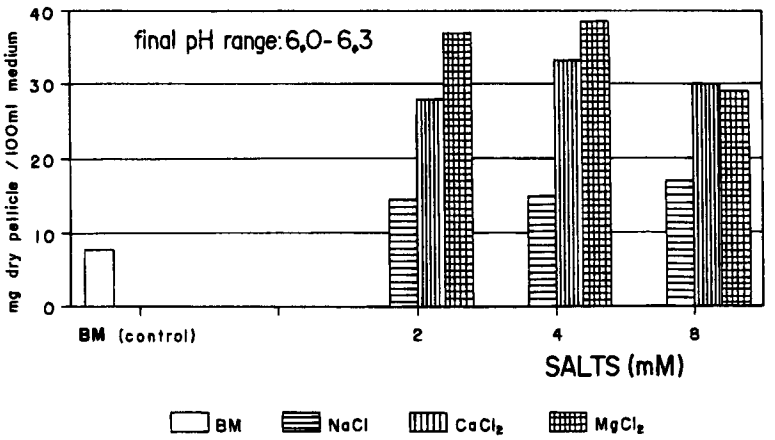

Fig. 1. Modulation of cellulosic membrane yields by strategic cations.

krpm (12,300 g) and lyophilization of the cleared and viscous supernatant. Guanosine (pyridine suspension) was derivatized with a stoichiometric amount of butyric anhydride for 12 h at room temperature and then dried in a Savant Speed vacuum machine with the application of heat.

RESULTS AND DISCUSSION

Culture media optimization was initially investigated via the dose–response for common, although strategic, cations owing to their important modulation of key enzymes of sugar activation/deactivation (e.g., phosphodiesterases). The best cellulosic pellicle mass increase was obtained with 2–4 m*M* calcium and magnesium chlorides (Fig. 1). Mixed at half-concentration, they act somewhat synergistically (results not shown), and were used in all subsequent experiments.

Ammonium dihydrogen phosphate (0.012 g/L) yielded the same cellulose equivalent to that obtained from a more expensive nitrogen source, namely, yeast extract (0.12 g/L) (Fig. 2). A better buffering condition against acetic acid action was also attained with this simpler nitrogen source. The particular effect of other sources like purines and pyrimidnes (Fig. 1) will be mentioned later.

CSL alone or in combination with tea (*C. sinensis*) infusions (Fig. 3) resulted in cellulosic membrane mass increases from 2- to 10-fold. Combinations of these complex nutrient sources resulted in additive effects (see the thicker and more uniform membrane in the fourth vessel from Fig. 4) and darker membranes, thus requiring more extensive washings.

An aqueous extract of previously organosolvent-extracted tea still stimulated cellulose production. Since pseudopurines were previously removed with the organosolvents, this gain is not explainable by caffeine/theophylline *(6)*. Therefore, beneficial tea components other than the pseudoxantines do exist and remain to be characterized.

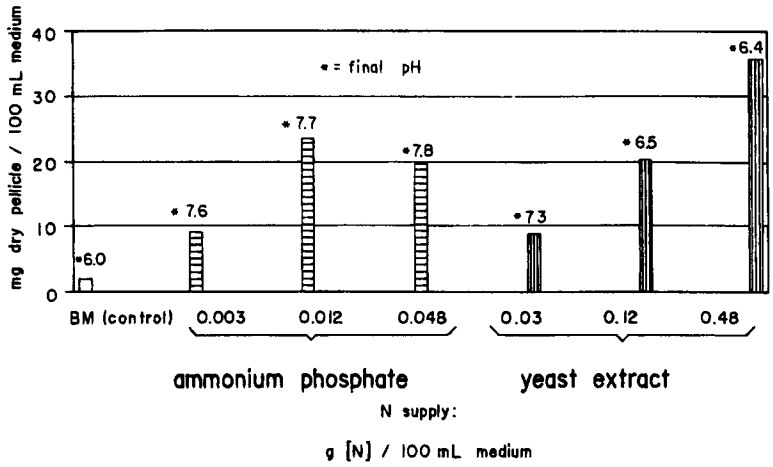

Fig. 2. The comparative effect of defined and undefined nitrogen sources for cellulose production.

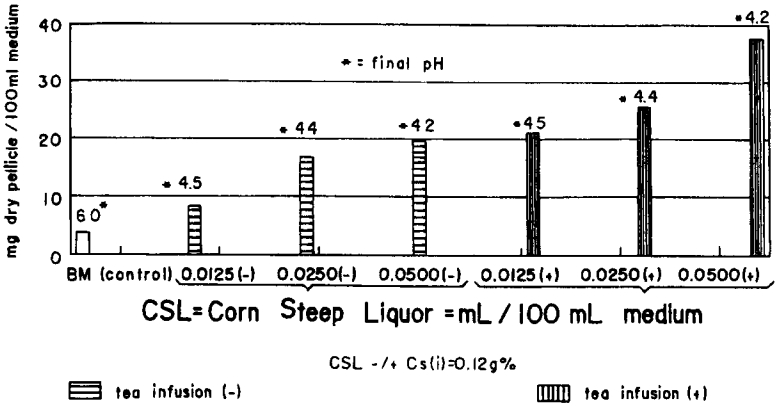

Fig. 3. Cellulosic membranes yield increase by CSL and/or tea infusion (CSi) additions.

CSL addition to the basic culture medium caused a severalfold increase in cellomembrane yield (Fig. 3). Interestingly, an anionic component from CSL (here recovered as its insoluble Ca^{2+} salt) produced a positive cellulogenic effect obtained from sodium phytate.

Since nucleotides play key roles in many biological processes *(12)*, some precursors, such as nitrogen bases or natural and lipoderivatized nucleosides, were examined as selective and limited (up to 5 mg/L) additions. Precursors of metabolically important compounds were chosen: guanine or guanosine because of the crucial positive role of $c(GMP)_2$ on cellulose synthetase and uridine as part of UDP-glucose, the activated glucosyl donor for the same enzyme. A stimulated basic medium was used as control (Table 1). Data indicated a positive, although limited, effect on cel-

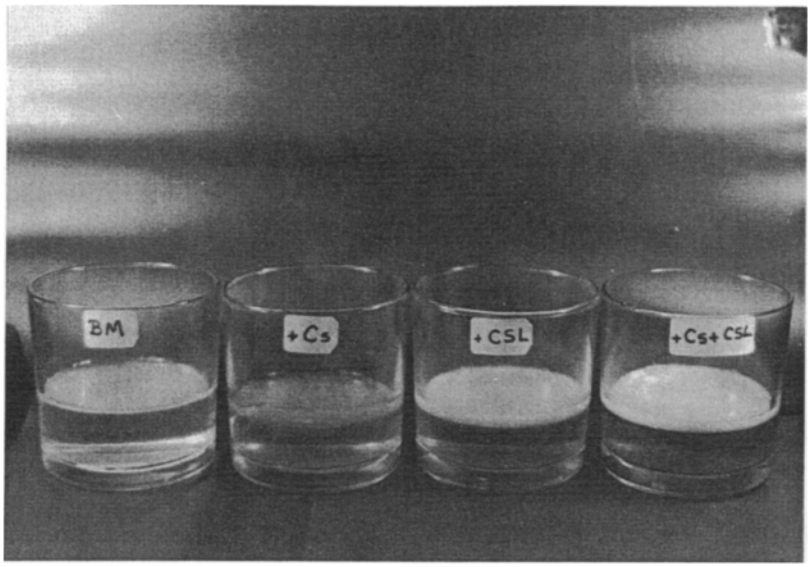

Fig. 4. Morphological aspect of cellomembranes from nonstimulated and stimulated culture media. (BM = basic medium; +Cs = addition of tea [*C. sinensis*] infusion; +CSL = addition of corn steep liquor; +Cs + CSL = both additions.)

lulose yield. Work is now in progress on long-chain derivatives (e.g., oleoyl) because of the possible toxic effects of free butyric acid.

The defined additions for other assays were phytic acid (sodium salt, pH adjusted to 6.8) and betaine, but the enhancement of cellulose yields was obtained only with higher inputs (0.5 and 0.05 g/L; Tables 1, 2, and 3). Interestingly, a fraction obtained from CSL as the precipitate from the addition of saturated $CaCl_2$ reproduced the effect of phytin. The particular effect of this cyclitol polyphosphate is difficult to explain in light of its chelating properties for Ca^{2+} and Mg^{2+}, but its potential benefit as precursor for phosphatidylinositol, an important component of biological membranes, cannot be discounted. Betaine may play the role of a membrane-reinforcer "osmolyte" *(13)*.

Since *Acetobacter* spp. may also biosynthesize other polysaccharides (e.g., the water-soluble acetan *14*), washing and/or pretreatment of the cellulosic membranes should resolve this issue. An alkaline bath treatment is the usual practice to obtain whitened membranes. Strong NaOH washing (5*M*, 6 h at room temperature with gentle agitation in a rotatory shaker) indeed produced such a desirable result. However, a dramatic effect of 3.5 times size reduction by this treatment (mass almost unaffected) was experienced by biofilms from both strains (Fig. 5). The new compact cellulose membrane architecture preserved some of its original flexibility, if maintained as hydrated material. Possible applications for these mercerized cellofilms are beyond the scope of this article, but ongoing physical analyses may shed some light on this aspect.

Table 1
Effect of Defined Supplements on *Acetobacter* Cellulosic Biofilm Production

		mg dry pellicle/100 mL medium	
		Acetobacter strain	
addition	concentration	"M"	"G"
BM #	(-)	119	74
BM + Na phytate	375 mg/L	148	123
Guanine	5 mg/L	129	93
Guanosine (GR)	5 mg/L	133	99
butyrylated GR *	0.25 mg/L	122	86
uridine	5 mg/L	125	89

#BM = basic medium = 40 g/L glucose, 1.25 g/L yeast extract, 0.25 g/L ammonium dihydrogen phosphate, 1 mg/L (each) caffeine + teophilline, 0.3 m*M* (each) Na^+, Ca^{2+}, Mg^{2+} chlorides, 5 mL/L ethanol.
*Derivative soluble in anhydrous methanol.

The expected nature of the polymer biosynthesized by both strains was then confirmed as cellulose using detergent-washed membranes in order to reduce any noise (entrapped cells and their multicomponents) in solid-state ^{13}C-NMR spectroscopy. The five-peak spectrum for Ax-M was similar to that previously reported for cellulose from the alga *Valonia (15)* and from crosspolarization/magic angle spinning (CP/MAS)-NMR *(16)*. This spectrum was minimally altered (Fig. 6) when an air-dried membrane was previously equilibrated overnight with a 1 g% solution of tamarind seed xyloglucan (Ts-XG) followed by extensive washing (unbound polysaccharide removal). Except for a small new peak (asterisk label) at $\delta = 97.1$ ppm from nonreducing -D-xylopyranosyl branching units of Ts-XG), no differences were noticeable since the Ts-XGT main -D-glucopyranosyl backbone is also cellulose.

SDS-washed cellulosic membranes, with (AxM-cel/Ts-XG) or without (Ax-M-cel) xyloglucan addition were also submitted to diffractometric analysis. The X-ray diffraction diagram of the control (Ax-M-cel) membrane is shown in Fig. 7. The concentric and complete-ring array

Table 2
Effect of Nondefined Supplements Related to Tea Infusion on *Acetobacter* Cellulosic Biofilm Production

		mg dry pellicle / 100 mL medium	
		Acetobacter strain	
addition	concentration	"M"	"G"
BM #	(-)	30	14
BM + caffeine theophylline	2 mg/L (each)	60	30
BM + tea infusion	.2 g/L	135	33
BM + tea aq. fraction *	2.0 g/L	60	45
BM + betaine	50 mg/L	53	24

#BM = basic medium = 40 g/L glucose, 0.125 g/L yeast extract, 0.1 g/L ammonium dihydrogen phosphate, 0.3 m*M* (each) Na^+, Ca^{2+}, and Mg^{2+} chlorides, 5 mL/L ethanol.

*Whole tea was exhaustively extracted with organic solvents of increasing polarity (iso-octane to methanol) and the final residue then extracted with boiling water to render the assayed fraction.

indicated a high degree of crystallinity, but no preferential orientation for the cellulosic microfibril both kind of samples (control; Ax-M-cel and Ax-M-cel/Ts-XG) were compared by scanning and reflection geometry (θ-2 θ plot), a lower amorphous content (Fig. 8; asterisk label) was detected after Ts-XG binding, but the same peaks with no change in positions indicated the preservation of the same planar spacings in the crystalline regions. The diffractometry tracing on the two main peaks (2θ-degrees = 14.5 and 22.76, corresponding to the 101 and 002 nomenclature for cellulose crystallites) fitted closely to those reported for 1% NaOH-boiled bacterial cellulose, namely, 2θ-degrees = 14.52 and 22.67 *(17)*, except for their similar intensities in our diffractograms. The third diffractometric data (peak 101; 2θ-degrees = 16.8), although coincident in terms of position, was less distinguishable, but the different sample pretreatments may be recalled (SDS washing, our case; alkaline boiling for 8 h, ref. *17*).

Table 3
Effect of CSL and Derivatives on *Acetobacter* Cellulosic Biofilm Production

		mg of dry pellicle/100 mL medium	
		Acetobacter strain	
addition	concentration	"M"	"G"
BM	(-)	11	14
BM + caffeine (#) theophylline	2 mg/L (each)	17	18
BM + CSL	10 mL/L	88	103
BM# + CSL	10 mL/L	104	112
BM# + Na phytate	0.5 g/L	37	32
BM# + CSL Ca2+ pp *	0.5 g/L	47	35

#All experiments with CSL or fractions contained the basal (2 mg/L) addition of pseudopurines.

*Fraction from CSL precipitated by the addition of a saturated solution of calcium chloride.

CONCLUSIONS

Growth of wild-type *Acetobacter* strains for the production of floating cellulosic membranes could be improved through the incorporation of simple and complex conutrients in the media for nonagitated cultures (e.g., cellulose mass doubling owing to 2 m*M* calcium ion). CSL proved a cheap and very productive addition. Slight or drastic changes in the cellulosic pellicles resulted from the incorporation of either structurally related hemicelluloses (e.g., xyloglucan) or from strong alkali mercerization (e.g., 5*M* KOH), respectively. These modified products may generate new (bio)technological applications other than the reported one as a temporary skin substitute in the case of human burns and other dermic injuries.

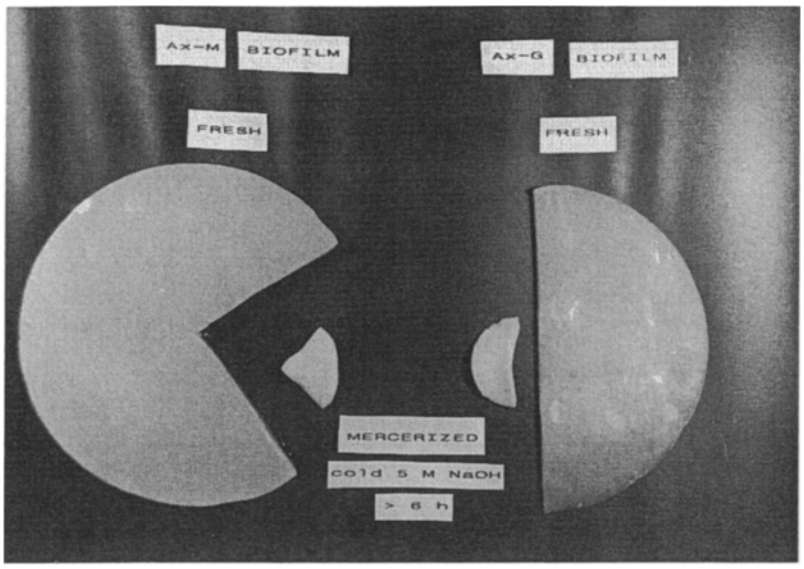

Fig. 5. Mercerization of fresh cellulosic biofilms (a quarter [Ax-M strain] or a half [Ax-G strain] of fresh cellulosic membranes was submerged in 5 *M* NaOH at room temperature for >6 h, and then thoroughly washed with distilled water before picturing).

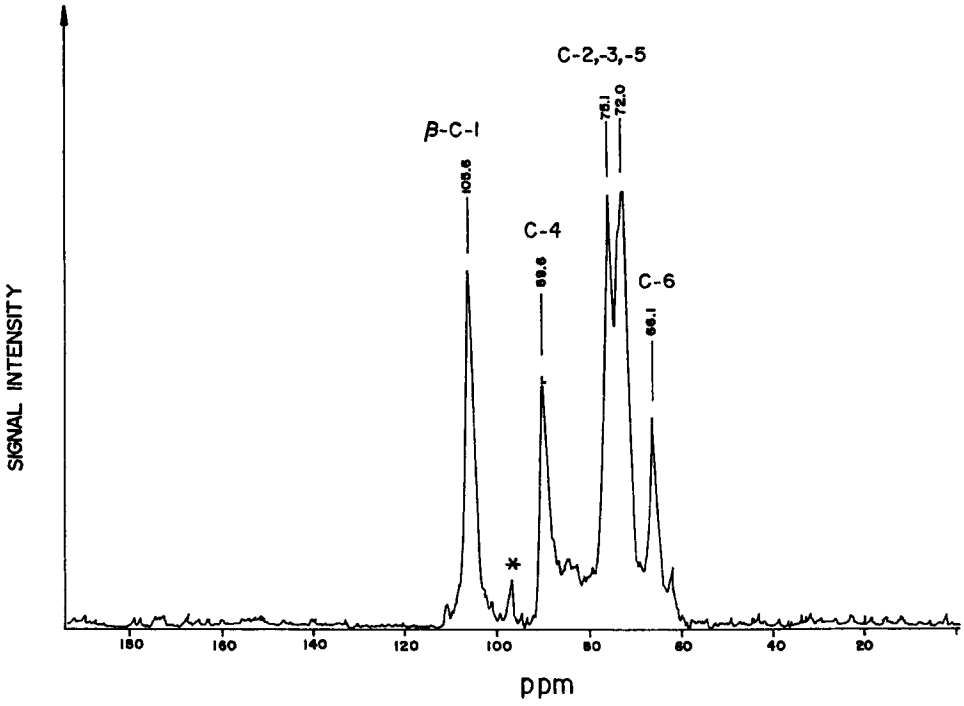

Fig. 6. Solid-state ^{13}C-NMR spectrum of an air-dried and powdered SDS-washed membrane (*) Before the spectral run, this particular air-dried membrane obtained from the Ax-M strain was equilibrated with xyloglucan and then thoroughly washed before comminution and drying.

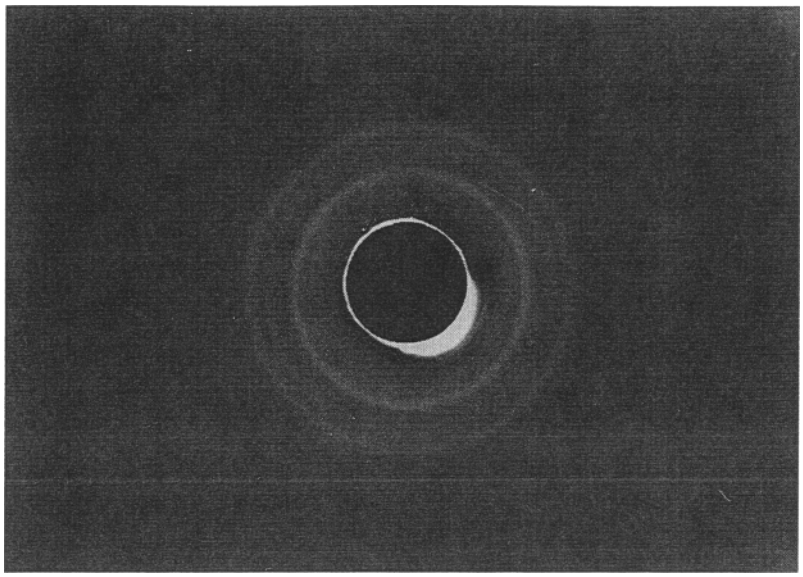

Fig. 7. X-ray transmission Laue diagram for an SDS-washed and air-dried membrane from the Ax-M strain.

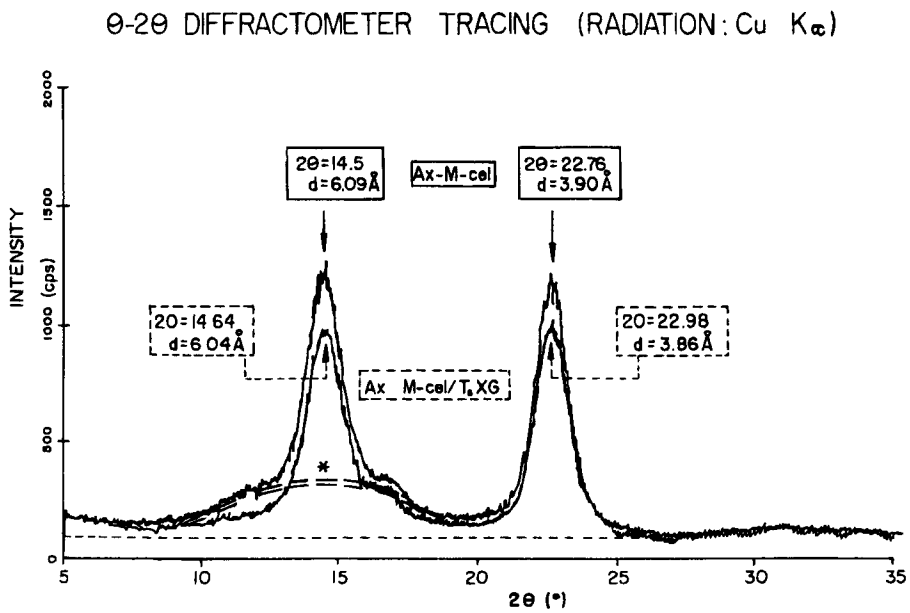

Fig. 8. Scanning and reflection geometry (θ2θ plot) from X-ray diffractometry of SDS-washed and air-dried membranes from Ax-M strain (upper tracing = membrane without addition of xyloglucan; lower tracing = membrane with addition of xyloglucan).

ACKNOWLEDGMENTS

Thanks are addressed to Brazilian funding agencies (CNPq/PADCT-SBIO and CAPES) and to A. C. Ramalho (UNICAMP; technical assistance with X-rays diffactometry), G. Yates (manuscript reading), and M. Lima (drawings).

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