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Acetobacter Cellulose Pellicle as a Temporary Skin Substitute

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ABSTRACT

A bacterial strain with morphological and biochemical properties close to *Acetobacter xylinum* has been cultured in nonagitated, inverted sucrose- and yeast water-based medium for the production of thick, smooth, and floating cellulosic pellicles. The cellulose content (>90%, dry weight, depending on the efficiency of water washing) and the β-D-homopolyglucan nature of these pellicles were assessed by physical, chemical, and enzymatic methods. The apyrogenic bacterial biomass, a minor component of the dried biofilm (BioFill®), is inactivated by ethylene dioxide. Once applied on exudating or bloody tissues, this biofilm displays several advantages as a biological dressing, and hence, it is valuable as a temporary skin substitute in the treatment of skin wounds, such as burns, ulcers, grafts, and as an adjuvant in dermal abrasions.

Index Entries: *Acetobacter;* cellulose pellicles; skin substitute; biological dressing.

INTRODUCTION

Cellulose biosynthesis in some acetobacteria has been known for more than 100 years (*1,2*). Cellulose membrane formation by *Acetobacter xylinum*, and its flotation, correlate with the carbon dioxide evolution

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from the fermented free sugars. The whole process is favored by an appropriate oxygen supply to the nonagitaged cultures, as opposed to shaken cultures that restrict cellulogenesis (*3*). The synthesis of a true cellulosic film by *A. xylinum* (*A. aceti* ssp. *xylinum*) (*4*), an unique anabolism amid bacteria, involves the extrusion of nascent β-D-glucan chains and/or partially crystallized microfibrils through a pore row disposed at the level of the bacterial cell membrane (*5,6*). This biosythetic model for the cellulose ribbon exportation and the regulation of the key enzyme, cellulose synthetase, by a cyclic guanyl-dinucleotide coupled to a specific phosphodiesterase has been established recently (*7,8*).

Bacterial cellulose is different from algal or plant homologous β-D-glucans (*9*), because the latter compose part of the cell-wall architecture (*10*). The natural plolysaccharide synthesized by *A. xylinum* displays characteristics of type I cellulose: parallel chains and offset plans (*11*). In vitro synthesis of cellulose can be also carried out with detergent-solubilized cellulose synthase, resulting in small fibrils of 17 Å in diameter (*7*). Some strains of *A. xylinum* also synthesize a xanthan gum-like exopolysaccharide (*12*), now designated as "acetan." Comparative studies on the balance of cellulose/acetan synthesis in selected strains of *A. xylinum* and naturally-derived mutants are in progress (*13*).

Artificial skin development is an important medical advance (*14,15*). We report a partial characterization of a cellulosic biofilm, produced by a wild type bacterial strain, with morphological, physiological, and biochemical properties of an *Acetobacter* sp., as well some of the current medical uses in the therapy of skin wounds.

MATERIALS AND METHODS

The strain being used is a wild type microorganism isolated from a decomposing homemade *Matricaria* sp. infusion by L. F. Farah (*16*). The similarity of the resulting pellicle with human skin inspired its medical application as a temporary skin substitute (*17*). Cultures of the strain are maintained at 28°C in solid or unshaken liquid media containing 5% glucose, 1% yeast extract, and 3% calcium carbonate (GYC medium) (*18*); stocks are transfered biweekly. Enriched cultures, when needed, were produced substituting 4% ethanol for glucose as the sole C source (*19*). The water soluble fraction of infusions, prepared from *Ilex, Paulinia, Camelia,* and *Coffea* spp. and malt extract, can also support *Acetobacter* growth instead of yeast water or extract. Scaled up cultures for the cellulose membrane preparation (*2*) (36–60 h at 28°C) were carried out in 5% sucrose that was partially inverted by baker yeast or mild H^+ hydrolysis during routine steam sterilization. Medium supplementation was provided by the yeast-water, generated in the previous step, after a brief boiling procedure.

Monitoring of total and reducing sugars and acetic acid was performed by the phenol-sulfuric method (*20*), alkaline copper method (*21*) and gas chromatography on a Porapak Q column, respectively. Optical micrographs were taken with an AFX-II device in a Nikon-Labophot microscope and electron micrographs with a Phillips-SEM 505 scanning electron microscope. Infrared spectra of acetone and vacuum-dried bacterial film, as well from its O-acetylated derivative, were recorded with a Perkin Elmer-Aculab M IR spectrometer. The X-ray diffraction diagrams were obtained with a Flat Laue camera using transmission geometry and Ni filtered Cu (Kα) radiation. The samples were placed in a specimen holder with mylar windows, where they could be kept wet during the exposure. Dry samples were mounted on a fiber specimen holder. Exposure time was generally 4 h, and the specimen to film distance was 42 mm. Enzymatic hydrolysis was performed using Celluclast® (Novo Industri A/S, Copenhagen, Denmark) or snail gastric juice hydrolases, following the general guidelines of a previous work (*22*). Trifluoroacetic acid (TFA) hydrolysis was carried out, as described by Albersheim (*23*), except that cellulosic films were preswollen in the concentrated acid (1:1, v/v) for 2 h at 95°C. Samples for thin layer chromatography (TLC) were run in Silica Gel 60 chromatoplates (Merck E. Merchag, Darmstadt, W. Germany) and gas liquid chromatoggraphy (GLC) using alditol acetate (OV-225 column) or per-silyl derivatives (SE-30) of mono- and disaccharide standards and samples (*22*). Assessment of asseptically water- or saline-rewet biofilms in skin treatments were conducted under the supervision of a dermatologist (J. A. Bichara) in patients suffering of skin wounds resulting from burns, ulcers, or facial dermabrasion. Parallel medical research data in outpatients, infirmaries, clinics, and hospitals were also compiled.

RESULTS AND DISCUSSION

The *Acetobacter* strain currently used is a Gram-negative rod- to ellipsoidally-shaped bacterium (Fig. 1), with a cell type and assembling pattern (individualized cells, pairs, and chains) resembling the characteristics and variability described for the *Acetobacter* genus and some *aceti* species (*18*). It also presents other attributes ascribed to the *aceti* sp. and *xylinum* ssp., such as reduction or loss of cellulose-synthezing ability (*24*) and cell pleomorphism or staining variability owing to cell aging or culture media composition. The consortium with baker's yeast does not disturb the bacterial cell growth or the extracellular cellulosic pellicle formation, probably owing to the bacterium's ability to oxidize yeast-generated ethanol. In view of the buildup of acetic acid, a pH drop to 2.1 results in yeast growth inhibition and overoxidation of the acid to CO_2 and H_2O.

The supplement influence on the volumetric bioconversion of free sugars was measured as mL of wet cellulosic mantle/L of culture media:

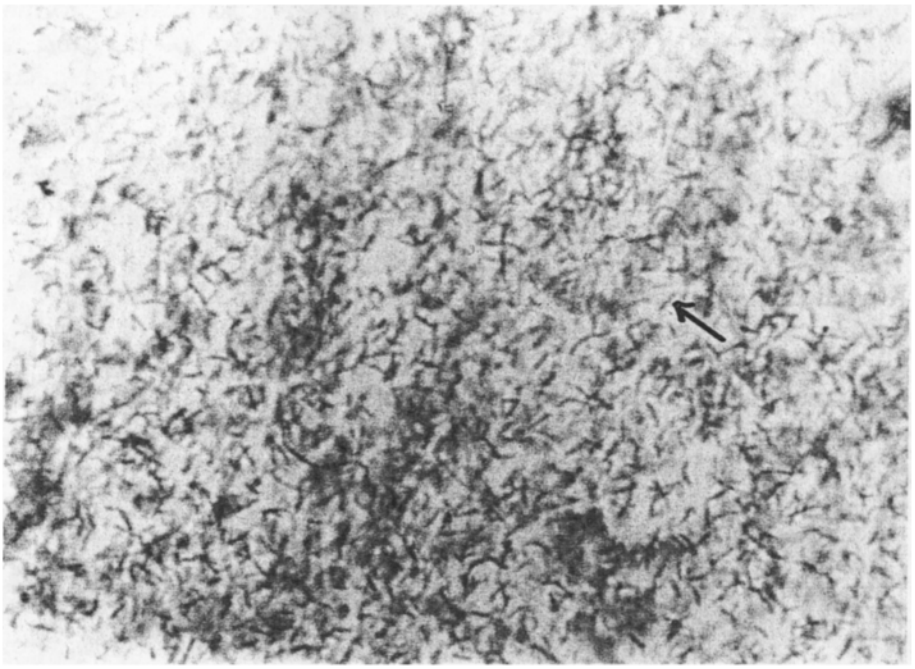

Fig. 1. Optical microscopy picture of a Coomassie R stained fresh biofilm from the *Acetobacter* wild type strain (2800×final magnification).

160 for yeast extract, 101 for *Ilex* extract, and 185 for a combination of both. Regarding the search of less expensive supplements, and in view of their heterogeneous composition, it is difficult to ascribe the benefit to a particular class of compounds or nutrients. Work is in progress to complete and report the fractionation of this and similar plant extracts in order to determine the value of inorganic salts, nitrogen-containing compounds, polymeric constituents, and trace elements.

The synthesized cellulosic network, which traps part of the bacterial cells even after thorough washing with water, is easily stained with dyes, such as Ponceau S (protein) and safranin (the counterstain for Gram negative microorganisms), or Congo Red. The biofilms become heavily fluorescent (UV light at 354 nm) after contact with CalcoFluor White ST, a probe reported to be selective for some β-D-glucans (*25*). Figure 1 shows a whole biofilm stained with Coomassie Brilliant Blue: the bacterial biomass is more intensely dyed owing to its protein content (one isolated cell is marked with an arrow). This staining profile is less visible on detergent (1% SDS) or 1*M* NaOH washed biofilms. If the native biofilm is washed with stronger alkali, i.e., 17.5 g%, as recommended for fractionation of α-, β-, and γ-cellulose (*26*), it experiences a dramatic shrinkage (approximately a 60% size reduction), whereas detergent washing results in cleaner and more transparent membranes, without shrinking.

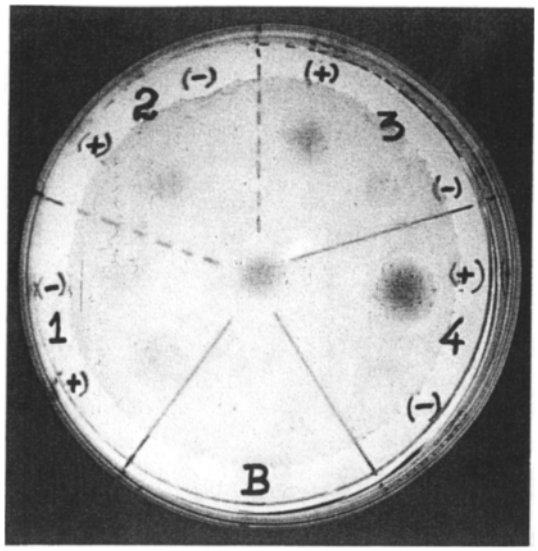

Fig. 2. Chromogenic plate test for the cellulosic nature of the *Acetobacter* biofilm: 1, 2, and 3 = different preparations of threefold diluted snail gut enzymes (native, sat. ammonium sulfate precipitate, and BioGel P-2 filtered, respectively); 4 = *Celluclast;* (−) = heat inactivated enzyme; and (+) = test with the active enzyme. B = zone of no enzyme application (D-glucose standard, 20 μg was applied in the center of the plate; other details in the text).

Slightly buffered biofilms (citrate–phosphate 10 m*M*, pH 4.8) are quickly depolymerized by cellulolytic enzymes from *Trichoderma* (Celluclast®) or the gut of the snail *Megalobulimus paranaguensis* (22). The final step of the breakdown of cellulose into cellodextrins, cellobiose, and then glucose may be easily seen if the digested biofilm is rebuffered at pH 7.0 (100 m*M* phosphate), containing a cocktail of glucose–oxidase plus peroxidase/aminoantipyrine (plate test in Fig. 2). Despite its lower cellulase concentration, treatment with the snail gut hydrolase complex also results in visible hydrolysis, owing to its higher cellobiase content. Furthermore, the almost pure cellulosic nature of the biofilm was suggested by the thin-layer chromatography of free sugars released by fungal cellulase action (Fig. 3, set A). Comparison of short- (lane S/A) with long-incubation times (lane L/A) indicates that the initial oligomeric spots are cellobiose + cellodextrins since they are converted to glucose. The same profile is seen with the native membrane (just washed with water; lanes S and L/W), but the free sugar resolution here is hampered by the contamination of the hydrolysate with other cell components. Under the same incubation conditions for the detergent-washed film (lane L/D), direct glucose release is preferential, possibly owing to a better performance of the β-glucosidase component of the cellulolytic complex. Accordingly, glucose appears as the product after strong trifluoroacetic acid (TFA) hydrolysis of the water-washed native biofilm (lane T/W). It was noted that 95°C treatment with

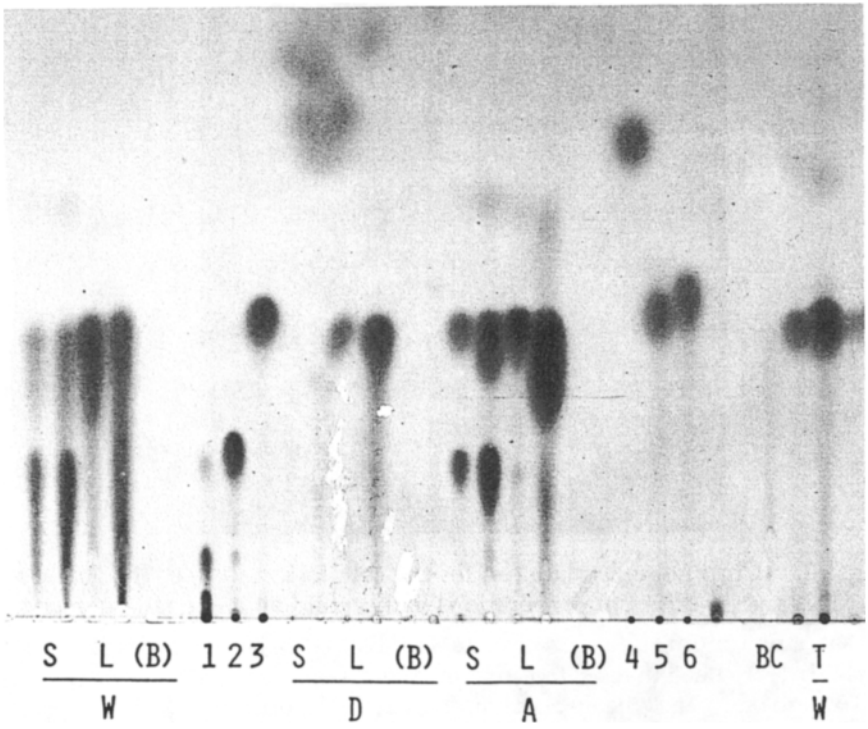

Fig. 3. Thin layer chromatography of sugars released from BioFill® after cellulolytic enzymes action (Celluclast®). W, D, A = water, detergent (1% SDS), and alkali (5*M* NaOH) washed biofilm substrates; S, L = Short (3 h) and Long (60 h) incubation times. (B) = enzyme "blank"; and T = Trifluoroacetic acid hydrolysis. Standards: 1 = cellodextrins; 2 = cellobiose; 3 = D-glucose; 4 = L-rhamnose; 5 = L-arabinose; and 6 = D-mannose. Plate: Silica-Gel 60, Merck; Solvent = isopropanol: ethyl acetate:water (85:5:10). Spray: orcinol/methanol/sulfuric acid.

concentrated TFA (diluted 1:2, owing to the moisure in the sample), does not lead to a complete pellicle dissolution, even after 6 h incubation. The contribution of the bacterial biomass weight (dry basis) probably cannot explain the partial retention of the membrane integrity. Infrared spectroscopy recorded for native, alkali, or detergent-washed biofilms, and their *O*-acetylated derivatives, disclosed some, but not all of the bands of maximal absorption for cellulose (*27*). For example, band type 2b at 891 cm^{-1} (resulting from deformational vibration of the axial ($C^{(1)}$–H bond) is noticeable. It is known that, in β-linked polyglucosans, the bands of types 1 and 3 (respectively for D-glucopyranosyl ring vibration and breathing) are reduced in intensity and sometimes absent altogether (*28*).

The search for trace or minor polymeric glycan components in the native biofilm was assessed in the alkali-solubilized fraction. For this, 20 g of a well-drained fresh pellice (previously defatted by boiling in chloroform–methanol) was extensively extracted with 5*M* sodium hydroxide,

containing 20 m*M* $NaBH_4$. The extract, filtered through a M porous glass membrane, was precipitated with 3 vol. absolute ethanol, producing 15 mg dry material. Free sugars, resulting from TFA hydrolysis, were converted to alditol-acetate derivates and analyzed by GLC. A monosaccharide profile glucose > arabinose > rhamnose > mannose is seen, and except for arabinose, the hexoses, and the deoxy-hexose are, incidentally, components of acetan, an acidic heteroglycan reported to be present in some wild-type and mutant strains of *Acetobacter xylinum* (*13*). Uronic acid acetan moiety, if also present in the hydrolysate, could not be detected as alditol acetate derivatives of mono- or oligosaccharide although in the "building blocking" process of biosynthesis, an acidic hepta-saccharide unit have been isolated (*29*). Glucose, as a major component, could be explained by the cellulosic "core" of acetan and less probably by some more easily hydrolyzable γ-cellulose contamination in the alkali extract. No explanation is available for the presence of arabinose since the only pentose reported for *Acetobacter* membrane is xylose (*11*), and only traces of the latter can be detected by GLC. It is worth mentioning that a differentiated cell wall structure was postulated for *Acetobacter* to explain the unusual cell resistance to lysis (*30*) and that this bacterium also perform the biosynthesis of β-1,2-branched glucan (*31*).

Scanning electron microscopy of osmium tetroxide-treated membranes are shown in Fig. 4. in frontal view for unused BioFill® (A) and the membrane after its spontaneous scaling from a skin wound treatment (B). In the latter, distinct layers of the cellulose pellicle are visible, bringing up some fragments of the epidermal crust. The ease in application of BioFill® as a therapeutical tool in skin wounds is exemplified in Fig. 5. Close adherence of the membrane to the wet wound bed follows, as is clearly visible in the central zone of the piece. The pellicle firmly sticks on glass surfaces as soon as the water excess is evaporated, and it does not allow liquid water crossing (Fig. 6), but does allow transfer of water vapor and gases, an important feature for current medical applications.

The growing cellulosic network displays a remarkable capability of water retention: 200 g of well-drained fresh *Acetobacter* biofilm (0.40 cm thickness) after drying results in only 1.35 g (average 0.05 mm thickness). Drying obviously causes dramatic changes in the porosity of the cellulose multilayer assembly, as well in the physical interaction between the polysaccharide individual chains. Microscopic inspection of a rehydrated film, pressed between two calibrated microscope covers, shows that the film experiences a very limited swelling not exceeding a doubling in thickness, compared with the eightfold reduction observed in the original dehydration step. The dried film displays almost no resistance to tear and moderately supports tension strength. The latter attribute increases dramatically in fresh rewetted cellulosic mantles.

Crystallinity and microfibril orientation is also affected by manipulation and physical/chemical treatments, as revealed by X-ray diffraction measurements (Fig.7A–D). The exhaustively washed biofilm (A, kept

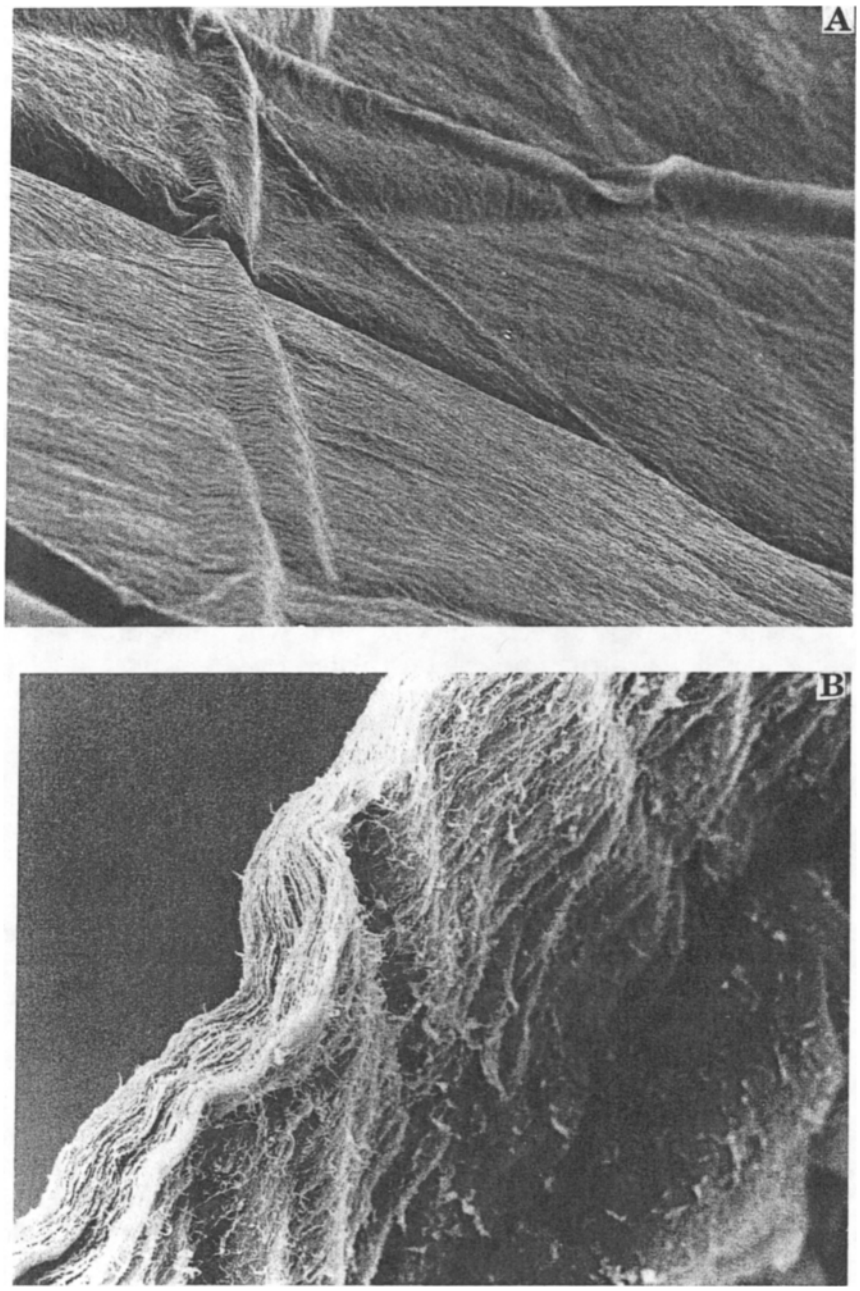

Fig. 4. Scanning electron micrograph of osmium tetroxide impregnated BioFill®. (**A**) Unusued biofilm (frontal view; 1200×final magnification); (**B**) Used Biofill®+fragments of the epidermal crust (2000×final magnification).

wet) discloses a diffuse halo in the X-ray diagram, a condition typical for amorphous cellulose. Upon a 10% stretching and drying, it acquires a preferential orientation for the crystallized regions (B), whereas the commercial product shows no preferencial orientation and some degree of crystallinity (C), this latter property increasing after alkali treatment and drying of the original wet pellicle (D, 10% stretched sample).

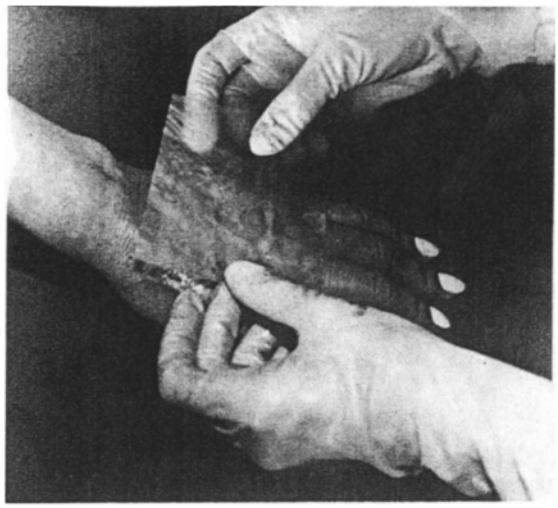

Fig. 5. Application of BioFill® to a human hand burn.

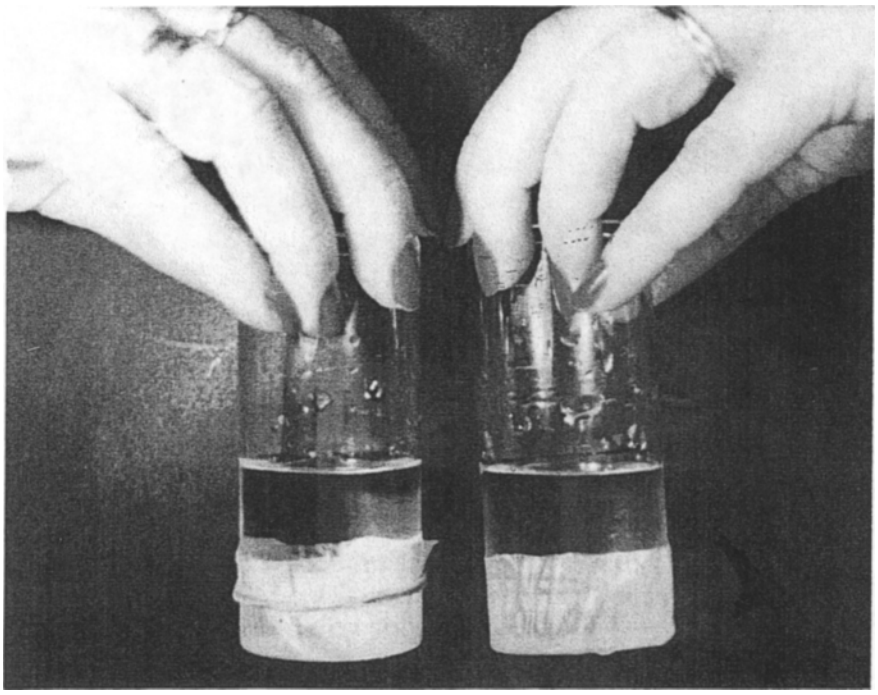

Fig. 6. BioFill® adherence and filtration properties (cylinder water column is 40 mL; for other details, *see* text).

BioFill® is experiencing an exponential use for several skin injury treatments, such as basal cell carcinoma/skin graft, severe body burns, facial peeling, sutures, dermabrasions, skin lesions and second degree burns, chronic ulcers, infectious epidermolysis, and both donor and receptor sites in skin grafts (*32–37*). These authors document the following advantages for BioFill® as a temporary skin substitute and biological dressing in

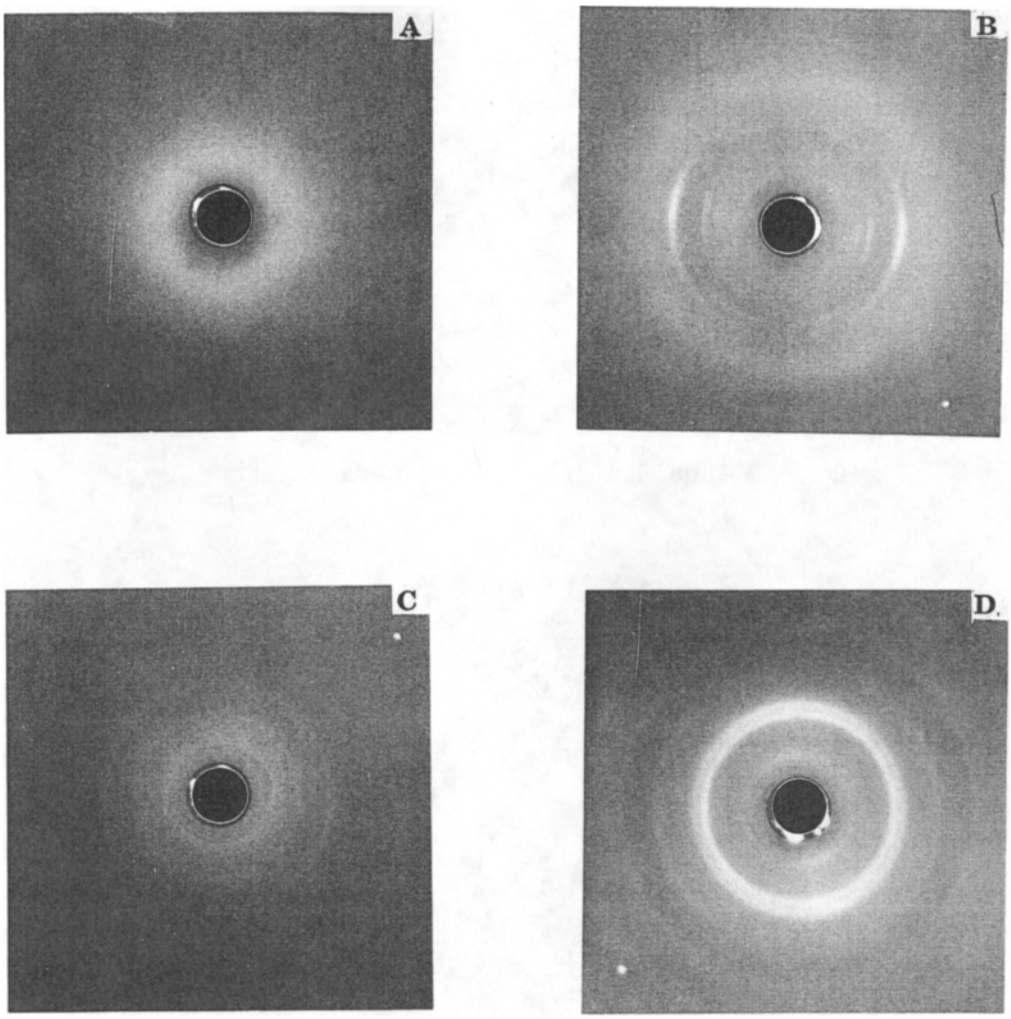

Fig. 7. X-ray diagrams of differently treated *Acetobacter* biofilms. (**A**) Exhaustively water washed biofilm (wet specimen). (**B**) Wet biofilm, 10% stretched (dried specimen). (**C**) BioFill® (commercial product; 8.5% moisture). (**D**) Wet biofilm previously treated with 17.5% alkali, 10% stretched (dried specimen). [A reference diagram was recorded for cotton cellulose (picture not shown)].

more than 300 treatments: immediate pain relief, close adhesion to the wound bed, diminished postsurgery discomfort, efficient barrier to pathogenic microbe installation/growth (reduced infection rates), easiness of the skin/wound inspection (transparency), faster healing, improved exudate retention (water, electrolytes, and proteins), expontaneous detachment following reepithelization, and reduced treatment time and costs.

Part of the same authors have indicated just one flaw, limited elasticity when application of BioFill® is made in areas of great mobility (e.g., perioral region).

CONCLUSIONS

Cellulose recovered from richer natural sources (e.g., lignocellulosics) requires drastic chemical treatments in order to ensure complete removal of lignin, hemicelluloses, and extractives. This results in a completely altered structure and, hence, the irreversible loss of advantageous native properties regarding the medical applications pursued in this work. Cotton cellulose is the natural source of highest purity, but its applications, though of marked usefulness, is limited to the well-established laboratorial and hospital routines. *Acetobacter* pellicle, a very intimate association of almost pure cellulose microfibrils and parts of the apyrogenic microbial biomass, owing to its peculiar physical, chemical, and biological properties, is an efficacious and improved tool for the treatment of several skin wounds. Further insights in the fine architecture of this kind of biofilm, the molecular nature of its minor constituents, and its biological effects are being undertaken to search for improvement of this very successful biotechnological advance and for possible alternative uses.

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