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Nature of Plant Stimulators in the Production of *Acetobacter xylinum* ("Tea Fungus") Biofilm Used in Skin Therapy

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ABSTRACT

Caffeine and related xanthines were identified as potent stimulators for the bacterial cellulose production in *A. xylinum*. These compounds are present in several plants whose infusions are useful as culture-medium supplements for this acetobacterium.

The proposed target for these native purine-like inhibitory substances is the novel diguanyl nucleotide phosphodiesterase(s) that participate(s) in the bacterial cellulogenic complex.

A better understanding of this feature of *A. xylinum* physiology may facilitate the preparation of bacterial cellulose pellicles, which are applied as a biotechnological tool in the treatment of skin burns and other dermal injuries.

Index Entries: *Acetobacter xylinum;* cellulosic pellicle; biological dressing; xanthine stimulation; "tea fungus"; caffeine.

INTRODUCTION

Following the first report on *Acetobacter* (cellulose) pellicle formation in 1886 (*1*), more than one century elapsed before the discovery of the

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regulatory mechanism involved in the bacterial cellulose synthesis (2–7). The latter authors showed the participation of an unusual diguanyl cyclic nucleotide as a positive effector of the *A. xylinum* cellulose synthase activity. The whole enzymatic complex also includes a specific guanyl cyclase, activated by Mg^{2+}, and a pair of specific phosphodiesterases performing a two-step degradation of the cyclic activator. The first esterase has its action blocked by Ca^{2+} (7).

Among the natural sources, *A. xylinum* has been reported as participating in formation of the so-called "tea fungus" (8), a term that probably arises from the bacterium's unique ability to synthesize a floating cellulose network that resembles a surface mold on nonagitated liquid media. *A. xylinum* mantle is also referred to, in lay terms, as "algal jam" or "zooglea" on home-made preparations. A more proper designation might be "schizoglea". On the other hand, the bacterium supports temporary consortium with other microorganisms (e.g., common yeast [9]), since its marked acidogenic metabolism on ethanol and/or free sugars guarantees a dominance at the log/late-growth phases. Lysis of the other microorganism may help the uptake of additional nutrients. Thus, the expression "tea fungus" might also be rationalized in this way. Recently proposed alternative environmental roles for the *A. xylinum* mantle were protection against UV radiation and enhanced colonization of sugar substrates, when competing with other microorganisms (10). Nutritional requirements for acetobacteria have been reported in relation to C and N sources, but the *xylinum* species was not included among the seven species studied (11). Growth on ethanol or sucrose may be useful in differentiation of species of *Acetobacter,* namely, *aceti, liquefaciens, pasteurianus,* and *hansenii* (8). No growth was reported for the species *hansenii* on ethanol or for species *pasteurianus* on sucrose. Not all strains of a given species were able to grow in the disaccharide, as seen for *A. aceti,* which may include, according to some authors, *xylinum* as a subspecies (12). *A. xylinum* has been reclassified as *A. hansenii* (8,13). Thus, a previous inversion of sucrose by yeast or even the lysed yeast presence may favor the growth of any *Acetobacter* strain unable to grow on sucrose. Mixed cultures of *Acetobacter* and *Candida* were reported (14). A defined synthetic medium was designed for growth and cellulose production by *A. xylinum,* and, except for the already used C sources (glucose, citric acid) and salts, it included nicotinamide (13), a key building block of redox coenzymes. The advantage of this medium is its efficiency for selection of auxotrophic mutants, and, less important from a basic research standpoint, the only disadvantage is its reduced efficiency for cellulose synthesis, compared to undefined media (15). We have reported the preparation of cellulosic biofilm using a wild-type strain of *A. xylinum* (here designated strain BF) on an industrial scale and for medical uses—for example, as a temporary skin substitute in burns and other injuries (9). This cellulosic pellicle and its medical applications may be seen as a successful biotechnological enterprise, since the

last surveys (1987–1989), based solely in 10 Brazilian dermatologists' groups, have reported hundreds of favorable medical applications (*9*, *16–20*). We predicted the beneficial role of certain plant extracts in cellulose synthesis by *A. xylinum* (*9*), when they were used as substitutes for such conventional and conutritional sources as yeast and malt extracts.

The present investigation is centered in the nature of compounds arising from such plants as *Camellia* (commercial tea), *Paulinia* ("guaraná"), *Coffea* (unroasted seeds), *Theobroma* (cacao), *Kola* or *Sterculia* (cola nut), and *Ilex* ("mate") and their possible role as positive effector(s) in cellulose synthesis by *A. xylinum*. These plants display, as a common feature, a high content of xanthine-based substances. Because of the reduced amount required as supplement to the C source, their infusions may be used as low-priced ingredients in medium formulation.

MATERIALS AND METHODS

Microorganisms

The most frequently used wild strain of *A. xylinum* was BF, and it was inoculated directly from fresh BioFill® biofilms (*9*). Strains JF, from a souring "guaraná" soft drink, and USDA 207, provided by R. Tanner, were kept in a $CaCO_3$-enriched medium as stock cultures and were used to confirm the stimulatory effect of pure drugs. Other conditions were described in the previous report (*9*), heavy inocula being adopted to shorten culture time to <5 at 28°C without agitation in a BOD 347-CD Incubator (Tecnal, São Paulo, Brazil). Unfavorable conditions for the growth of yeast and other microorganisms were ensured by the natural production of acetic acid by *A. xylinum* and the inclusion of 0.1 mL% of Mycostatin® (Squibb Industria Quimica SA) and 0.5 mg/mL of Benomyl® (du Pont) in the media. Fruitfly access to the open fermentation vessels was avoided by covering them with napkins.

Plant Active-Compounds Assessment

Fermentations were carried out in half-filled, 200-mL, open cylindrical vessels, C sources being either 5% glucose, sucrose, or yeast-inverted sugars supplemented with 0.5 mL% of ethanol. Growth and floating cellulosic mantle production proceeded without agitation of the cultures. Plant extracts (infusions) were prepared keeping each source (1.2 g of dry leaves or seeds) in contact with 1 L of freshly boiled distilled water for 30 min after filtration through a compact layer of glass wool. No particulate matter was visible in the resulting warm infusions, the solid dry weight of which accounted for <300 mg/L, most of the material being polysaccharide. Concerning culture-media formulation, the composition of these infusions (and yield, on a dry basis) ensured neither a significative rein-

forcement to the generous load of free sugars nor an excess of N and P sources. Part of the plant infusions, as known duplicative volumes and prior to inclusion in the fermentation media, was alternatively extracted with chloroform with or without pH adjustment in order to enhance or to reduce the xanthine content of the final aqueous solution to be used as media supplement. In order to avoid further dilution of the infusions, other media components were added as solids or from 10 concentrated solutions to 1.1× concentrated infusion. Yeast extract (0.005–0.01 g%) was included in the media in order to avoid too much reduced cellulosic membranes in the control experiments. ^{14}C-(U)-Glucose pulse-chase was carried out when all cultures presented visible growing membranes (48–60 h), hampering the excessive turnover of radioactivity to components other than cellulose.

Optical and Electronic Image Recording

Images from the cellulose network and bacterial biomass were recorded as described (*9*). In the case of optical microscopy, the material was previously dyed with a 10 mg/mL solution of Calcofluor white M2R in order to render cellulose fluorescent to UV light (*21*).

Analytical Procedures

Paper chromatography for radiolabeled free sugars was carried out on Whatman 3MM filter paper with ethyl acetate/isopropanol/acetic acid/methanol/water (10/2/1/4/8, v/v) following pretreatment of "cold" or ^{14}C-labeled membranes with boiling 5*M* KOH, buffering at pH 4.8, and their extensive fungal cellulolysis with Celluclast® (Novo Nordisk do Brasil, Araucaria-PR). Glucose, cellobiose, and traces of celloligosaccharides were made visible with alkaline silver nitrate and their ^{14}C-labeled fraction measured by liquid scintillometry in Bray's solution (*22*). GLC for xanthines, without previous derivatization, was performed in a Varian-Intralab 3300 gas chromatograph equipped with a capillary SE-30 column (J & W Scientific Inc.) and FID detector. This was confirmed by GLC-MS on a PONA dimethyl-silicone capillary column (Hewlett-Packard) in a trimodular 5890/5970/ChemiStation from the same supplier. Mass spectrometry was carried out using the electron-impact mode. Both columns were operated isothermically at 240 °C, using N_2 and He as carrier gases, respectively. UV spectra of chloroform-soluble plant extracts, after pretreatment with dilute HCl and redissolution in methanol, were recorded in a Varian-Intralab DMS-80 spectrophotomer. Plant infusions, after being made alkaline, were extracted with chloroform, and the resulting residues, containing the xanthines, were acidified with HCl and resolubilized with methanol. These materials were submitted to a chromogenic plate test using an inexpensive modification of the murexide reaction (*23*): addition to equal parts of $NaClO_4$ (< 10% of active Cl_2) and fuming HCl. Depending on the

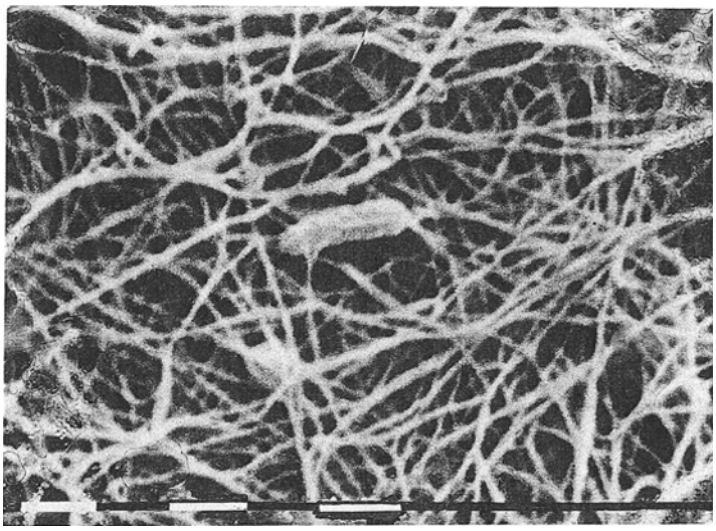

Fig. 1. Scanning electron micrograph (SEM) of an *A. xylinum* impregnated biofilm (strain "JF"); final magnification = 20,000.

amount of the material assayed, an orange-reddish color developed upon heating at 100°C.

RESULTS AND DISCUSSION

Growth of all strains of *A. xylinum* led, after 72 h at 28°C, to the formation of a compact and floating pellicle, provided that the medium formulation was favorable for cellulose biosynthesis. Viewing the membrane network by TEM of large magnification (Fig. 1; 20,000×) allows one to observe cellulose microfibrils and some trapped bacterial cells, which remain even after extensive water washings and membrane sequeezing. Native, detergent-, or alkali-treated membranes strongly took up CalcoFluor. The respective fluorescences under UV light (results not shown) were proportional to the cellulose-layer thickness and, hence, to the amount of synthesized cellulose in each membrane.

Comparative growth of *A. xylinum* in the free-sugar media, separately supplemented with reduced amounts of extracts from different families of plants (Sterculiaceae, Sapindaceae, Theaceae, Aquifoliaceae, Rubiaceae), resulted in a noticeable enhancement of the respective cellulosic membranes. The best stimulation, at room temperature, was obtained with tea infusions (*Camellia sinensis*) at longer cultivation (>7 d), and with "guaraná" (*Paulinia cupana*) at shorter times (<5 d). The effect can be more properly observed in the isolated and extensively washed pellicles (Fig. 2). The respective gravimetric data, after lyophylization, are shown

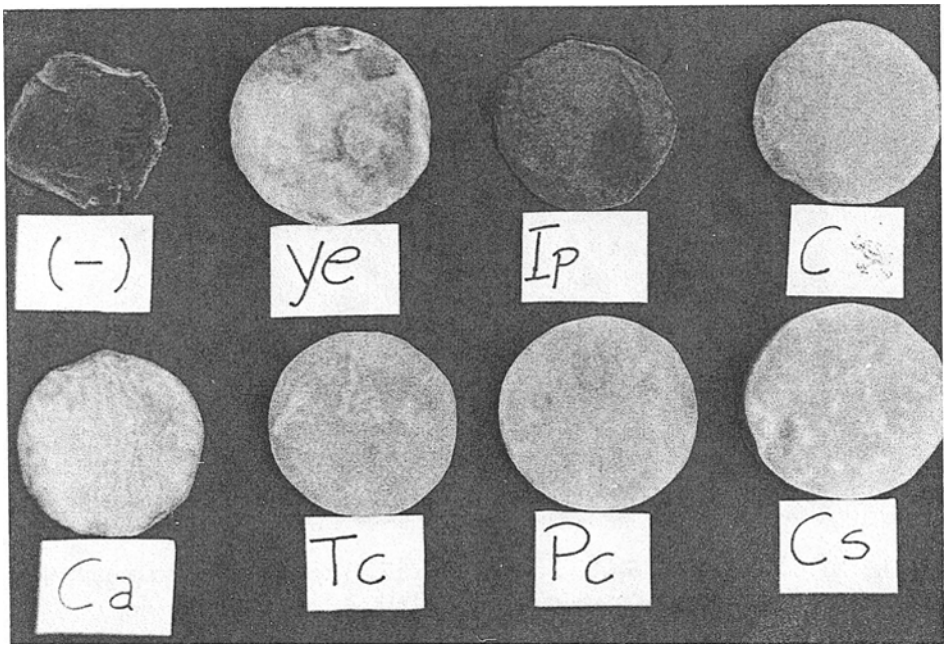

Fig. 2. Comparative aspect of processed *A. xylinum* membranes. The floating biofilms from the separate and nonagitated liquid cultures of strain "BF" (15 d at room temperature) were removed, thoroughly washed with water to remove loosely adhered cells and residual medium material, drained of excess water, and photographed. (−) control—basic medium without plant infusion addition. Supplementations as follows: ye = yeast extract (0.12g%); Ip = *Ilex paraguayensis* ("mate"); C = *Kola nitida* (cola nut); Ca = *Coffea arabica;* Tc = *Theobroma cacao;* Pc = *Paulinia cupana* ("guaraná"); Cs = *Camellia sinensis.*

in Table 1 (experiment A). These plant infusions (that is, the solubilized part of 120 mg of whole material/100 mL of solvent) resulted in variable residual matter upon solvent evaporation. Analysis for carbohydrate, in the case of tea infusion, indicated <30 mg%. Most of this content was polysaccharide material, a noncompetitive C source for the 5-g% load of free sugars in culture media. Protein was barely detected in 10%-trichloroacetic-precipitated material free of interfering low-mol-wt phenolic compounds. Contribution of nitrogenous compounds other than protein (except for those mentioned further on) were not evaluated. On the other hand, all tested plants contained a variable amount of xanthine-related bases, as detected in the respective chloroform extracts (e.g., about 2 mg in each 100 mL of tea infusion). Since these kinds of substances display biological activity (e.g., inhibition of the enzyme performing "second-messenger" degradation in glycogen metabolism [24]), there was clear evidence of a regulatory role for them in *A. xylinum* cellulose synthesis, which is also mediated by cyclic nucleotides (*6,7*). In order to check this hypothesis by comparison with untreated tea infusion (a; control), three

Table 1
Effect of Native and Partially Decaffeinated Plant Infusions in *A. xylinum* (strain "BF") Cellulosic Membrane Production

Additions or modifications to basic medium[a]	Wet (drained) weight, g%[b]	Dry (lyophilized) weight, mg%[b]
A[c]		
None	1.2	7.9
ye (yeast extract)	20.6	217.5
Cs (tea)	35.3	310.4
Pc (guaraná)	32.7	295.5
Ca (coffee)	14.0	104.2
Tc (cacao)	11.6	78.5
Cn (cola nut)	8.6	71.9
Ip ("mate")	7.5	75.2
B[d]		
Untreated tea infusion	—	44.8
Native pH; $CHCl_3$ extracted	—	9.1
HCl (pH <3); $CHCl_3$ extracted	—	23.7
NaOH (pH >11); $CHCl_3$ extracted	—	4.5
C[d]		
Untreated tea infusion	3.3	30.7
CHCl extracted	0.7	10.6
$CHCl_3$ extracted; reconstituted	3.2	29.6

[a]Basic medium composition is as follows: 5 g% (inverted) sucrose in A and B; 5 g% glucose in C. Yeast-based supplementations: A=0.01 g% yeast extract; B and C=yeast water (replacing yeast extract on the same dry basis); 0.5 mL% ethanol in all experiments.
[b]Per 100 mL of liquid and nonagitated culture medium.
[c]Reduced inoculum; larger vessels (400 mL of medium); growth at room temperature for 15 d.
[d]Usual inoculum, vessel size, and incubator (*see* Materials and Methods section); crop of membranes at 84 h (experiment B) and 72 h (experiment C).

portions were pretreated by chloroform extraction as follows: (b) at the original pH, 5.6; (c) at an acidic pH (0.01*M* HCl); and (d) under basic (0.01*M* NaOH) conditions, the latter two then being neutralized. An equivalent amount of NaCl was added to samples (a) and (b). Gravimetric data for the resulting cellulosic pellicles using these modified tea infusions are shown in Table 1 (experiment B). It was clear that the pH increase, favoring the extraction of alkaloid-like compounds by chloroform, was also proportionally unfavorable for cellulose synthesis. On the contrary, low-pH chloroform extraction (pH<3) left an infusion resulting in a membrane weight equivalent to half of that of the untreated infusion. Another experimental variation was to repeat the extraction with chloroform at the natural pH as duplicate runs (Table 1; experiment C). Reincorporation of residual matter into the growth medium after solvent evaporation resulted in complete recovery of cellulose synthesis. The use of control (untreated) and

pretreated infusions as culture-media supplements strongly indicated that most of the stimulatory compound(s) were extractable with chloroform under a neutral or basic condition. More-polar solvents (e.g., methanol) also removed the active compound(s) from infusion residues, but led to larger masses of extracted material, thus making it difficult to interpret the data. The residue from chloroform extract (under alkaline conditions), as well as the pure caffeine, gave positive reactions to the modified murexide test. UV spectra of the same samples displayed the same λ_{max} (about 270 nm). Confirmation of the structure for this positive regulator in cellulose biosynthesis by *A. xylinum* was followed by GLC on a SE-30 column (R_T for caffeine, theobromine, for theophylline standards equal to 12.7, 12.9, and 14.3 min): *Camellia* infusion furnished a single component with an $R_T = 12.2$ min. A similar result was obtained for *Paulinia* (with a larger peak area for the same R_T); *Coffea* and *Theobroma* presented, besides the major fast peak, other minor components, two of them coincident with the theobromine and theophylline standards. Definite identification of the major purine was achieved by GLC–MS: its trimethylxanthine nature was corroborated by an R_T coincident with that of the caffeine standard (12.7 min) and an e.i. fragmentation pattern almost identical to the literature report (*25*): 194 > 109 > 67 > 55 > 82. Despite the net and dominant procellulogenic effect of caffeine (trimethylxanthine) in all plant infusions used herein, the minor analogous dimethylxanthines (theophylline and theobromine) may be having an ancillary positive effect, at least in the cases of "guaraná" and cacao, as seen in their multipeak chromatographic profiles (results not shown). Incidentally, a more efficient stimulation was observed for *P. cupana*, using shorter culture times. This is in accordance with its higher content in caffeine (4.49 g%) along with five other xanthines and a small amount of free guanine (*26*), the pyrimidine base involved in building up the cellulose synthase cyclic nucleotide activator (*7*). Remaining to be explored is the greater yield of cellulose membrane with longer culture time using *Camellia* infusion, despite its somewhat lower content of caffeine. Other minor and polar components of these plant infusions must play a synergistical role to xanthines. Calcium and magnesium content in the ranges of 30–68 and 90–270 μg% were found in the presently used plant infusions. Phosphate was also an important component in culture media, and this agrees with a previous report for a minimal defined medium (*13*). In fact, we confirmed the positive contributions of these three substances to cellulose pellicle formation either as isolated or as combined additions, and keeping 0.12 g% yeast extract as a supplement (the latter fully soluble, as compared to tea in the same dry basis).

Our findings for xanthine activation of the cellulogenic complex from *A. xylinum* were finally confirmed by experimentation with pure xanthine drugs. Their positive effects, separately or in combination, are reported in Table 2. Radiolabeling (brief pulse-chase) with ^{14}-C-glucose indicated that cellulose was the single significant glycan being synthesized under

Table 2
Effect of Pure Xanthine Drugs in *A. xylinum*[a] Cellulosic Membrane Production

Addition to the basic medium[b]	mg%[b]	Wet (drained) weight g%[c]	Dry (lyophilized) weight, mg%[c]
None	—	2.0	30.0
Caffeine	0.6	8.1	151.3
	0.3	6.6	106.4
	0.15	5.1	90.7
	0.075	4.1	68.6
Theophylline	0.6	5.3	117.3
	0.3	4.3	97.8
	0.15	3.8	60.2
	0.075	3.5	58.0
Caffeine + theophylline	0.15 + 0.15	4.8	118.9

[a]Table data recorded from strain "BF"; effects confirmed in other strains.
[b]BM = 5 g% (inverted) sucrose; 5 mg% yeast extract; 0.5 mL% ethanol.
[c]Per 100 mL of liquid and nonagitated culture medium.

xanthine stimulation. Use of a potent cellulase (Celluclast®) on alkali-pretreated membranes led to recovery of almost all incorporated radioactivity as glucose and cellobiose (ratio of percentages of radioactivity = 60–70/40–30, taking average data for the three different strains used).

Caffeine has been described as a noncompetitive inhibitor of dog-heart phosphodiesterase (*27*). A similar action was ascribed to theophylline and other metilxanthines (*28*). The net effect of these drugs was, thus, the enlargement of the physiological half-life of "secondary messenger(s)." Our results pointed strongly to this situation in *A. xylinum*, the methylxanthines most probably blocking the action of the specific diguanyl cyclic phosphodiesterase(s) and then avoiding or postponing the normal "switch off" of the active cellulose synthase. Work is in progress on this subject and to evaluate the contribution of other low-mol-wt components from plant infusions for an actively anabolizing bacterial biomass, despite their reduced concentrations in the 0.12%-based infusions.

CONCLUSIONS

The historical statement of "tea fungus" adopted for *A. xylinum* was satisfactorily clarified. Activators for the bacterial cellulogenic complex and arising from *Camellia sinensis*, as well as from other, different plants, were identified as caffeine and related compounds (theophylline, theobromine). The suggested target(s) for these methylated xanthines were the unusual diguanyl cyclic phosphodiesterase(s) whose inhibition favors the cellulose biosynthesis. The modern medical biotechnology has accepted

artifical skins as a valid prospect (*29*). Several properties advantageous for its use as a temporary skin substitute were recognized for BioFill®, a competitive product based on a *A. xylinum* cellulosic biofilm (*9*). Its successful application by dermatologists and plastic surgeons in more than 400 cases (*9,16–20*) included human second- and third-degree skin burns, skin grafts, face peeling, infectious dermolysis, tatoo abrasions, trophic venous and chronic ulcers, and Hansenian legs. Histological and histometric studies in the repairing process of rat skin were also conducted with and without application of Biofill®. Enhanced proportions of fibroblasts, collagen, blood vessels, and granulation tissue were seen in the healing wound as a result of the cellulosic pellicle use, and the reepithelization reached completion after 21 d (*30*). Our interest remains focused on the acquisition of a better understanding of *A. xylinum* phisiology and in its optimized growth/cellulose production.

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