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MANNOGLUCOGALACTANS FROM THE CELL WALLS OF *Penicillium erythromellis*: ISOLATION AND PARTIAL CHARACTERISATION*

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ABSTRACT

Cell-wall material from *Penicillium erythromellis* was extracted with M sodium hydroxide at 20°. After neutralisation and dialysis of the alkali-soluble material, two polysaccharidic fractions were isolated, namely, a β-glucan (glucose, 49%) containing galactose (6.9%), xylose (3.7%), mannose (1.9%), and protein (9%), and a glucogalactan (glucose, 15%) with galactofuranosyl residues (39%), containing mannose (5.3%) and protein (18%). Borate anion-exchange chromatography of the glucogalactan gave five polysaccharides, the relative amounts of which, in order of elution, were 1.5:1.0:2.4:7.1:2.1. When the three polysaccharides with the highest carbohydrate content were subjected to methylation analysis, one was shown to be a (1→4)-α-D-glucan with branch points at positions 6 (12.2%), and glucopyranose (5.8%) and galactofuranose (3.4%) as terminal residues, and the others to be β-glucogalactans with different proportions of galactofuranose and glucopyranose residues. The galactofuranosyl residues were (1→2)-linked with branch points at positions 6, and galactofuranose as terminal residues. The glucopyranosyl residues were mainly (1→4)-linked with glucopyranose as terminal residues.

INTRODUCTION

Most studies of *Penicillium* cell walls have been concerned with the chemical analysis of either complete or fractionated hyphal cell-wall material. The cell walls of *P. notatum*[1], *P. chrysogenum*[2,3], *P. roquefortii*[4], *P. italicum*, *P. digitatum*[5], and *P. rubrum*[6] are made up of a glucan, with traces of galactose and mannose, chitin, and protein. However, the isolation and the structural features of *Penicillium* cell-wall polysaccharides, other than chitin, have seldom been reported. Leal *et al.*[7] found two different types of cell-wall polysaccharide in the alkali-soluble fraction of several species of *Penicillium*. In group A (*P. chrysogenum, P. oxalicum, and P. expansum*), the alkali-soluble material was partially characterised as an α-glucan

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with minor quantities of mannose and galactose[8]. In group B (*P. allahabadense, P. erythromellis, P. islandicum,* and *P. zacinthae*), the alkali-soluble fraction contained mainly a galactofuranan[9,10]. Galactofuranose residues are present in the cell-wall and extracellular polysaccharides of *P. charlesii*[11-13] and in the cell wall of *P. ochro-chloron*[14], and they seem to be antigenic determinants in some *Penicillium* species[15].

Grisaro *et al.*[5] suggested that the alkali-soluble fraction from *P. digitatum* and *P. italicum*, composed mainly of glucose and galactose, might be a mixture of homo- or hetero-polymers.

Since the molecular structure of *Penicillium* cell-wall polysaccharides has not been studied in detail, our main objectives were to find out whether the alkali-soluble fraction from *P. erythromellis* contained a heteropolysaccharide or a mixture of polysaccharides, and to determine their structures.

EXPERIMENTAL

Micro-organism and culture media. — *P. erythromellis* Hocking, strain 7284, was obtained from the collection of Dr. Ramírez (Centro de Investigaciones Biológicas, C.S.I.C., Madrid, Spain). The micro-organism was maintained on slants of Bacto potato dextrose agar supplemented with 1 g/L of Bacto yeast extract (Difco).

The basal medium used for mycelial growth has been described[16]. The medium, dispensed in 1-L portions into 2-L Erlenmeyer flasks, was autoclaved at 120° for 15 min. The flasks were inoculated with 1 mL of a conidial suspension (10^5 conidia), and incubated in an orbital incubator (Gallenkamp IH-465) at 25 ±1° and 150 r.p.m.

Cell-wall preparation. — The cultures were harvested after 17 days (medium phase of growth) and filtered through cheesecloth, and the mycelial mat was washed with distilled water (3 × 500 mL), then freeze-dried, powdered, and stored desiccated. Dry, powdered mycelium (60 g) was stirred with 4 L of aqueous 1% sodium dodecyl sulphate, containing 0.02% of sodium azide, at room temperature overnight. The cell-wall preparation was collected on filter paper, dispersed in distilled water, and disintegrated in a Sorvall omnimixer followed by treatment with ultrasound[10]. The cell-wall material (c.w.m.) was virtually free from contamination with cytoplasmic material and did not stain with Coomassie Blue when observed by phase-contrast microscopy. The clean cell walls were freeze-dried, extracted with ether in a Soxhlet for 20 h, and stored desiccated.

Fractionation of cell-wall material. — Dry c.w.m. (25 g) was extracted twice with M NaOH (1.5 L) for 2 h at room temperature. After centrifugation, the supernatant solutions were combined, the pH was adjusted to 6.0 with hydrochloric acid, and the solutions were then concentrated under reduced pressure and exhaustively dialysed against running tap water. The dialysate was concentrated to a small volume and kept frozen (F1). A sample was freeze-dried for analysis.

F1 was thawed and centrifuged to give a supernatant solution (F1S) and a

precipitate (F1P). A sample of F1S and all of F1P were freeze-dried for analysis. The remaining F1S was kept frozen.

Isolation of polysaccharides from F1S. — A solution of a portion (~525 mg) of F1S in 30mM sodium borate (pH 8.5, 27.5 mL) was added to a column (35 × 2.5 cm) of DEAE-Sepharose CL 6B (borate form) and eluted first with 30mM sodium borate (150 mL), then with a linear gradient of sodium borate (30mM→1.2M, 600 mL), and finally with 1.2M sodium borate (610 mL). Fractions (5 mL) were collected, and monitored for carbohydrate by the phenol-sulphuric acid method[17]. Appropriate fractions were combined, acidified with conc. hydrochloric acid, dialysed, concentrated to small volume, and freeze-dried. The absence of borate was checked in aqueous solutions of the combined samples with a curcumin reagent[18], using boric acid as standard.

Chemical analysis. — Total hexose was determined in the polysaccharides by the anthrone procedure[19], with D-glucose, D-galactose, and D-mannose as standards.

For the analysis of neutral sugars, the polysaccharides were hydrolysed either by 0.05M H_2SO_4 for 5 h at 100° (partial hydrolysis) or Saeman hydrolysis[20] for 2.5 h. The products were converted into the alditol acetates by a modification[10] of the method of Selvendran *et al.*[21] and analysed[16] by g.l.c. on a column of 3% SP-2340.

Protein was determined colorimetrically[22] on solutions of samples in either M NaOH or distilled water, with bovine serum albumin as standard. 2-Amino-2-deoxyglucose was determined colorimetrically[23] after hydrolysis with 6M HCl for 4 h at 100°, and the chitin content was calculated as the "anhydro-2-acetamido-2-deoxy-D-glucopyranose" equivalent. 2-Amino-2-deoxyglucose was identified with an LC-7000 Biotronik Amino Acid Analyzer, data being acquired on an SP 4100 Spectra Physics computing integrator.

Phosphate ion was determined by the method of Rand *et al.*[18] in samples hydrolysed with 2M HCl for 2 h at 100°.

Optical rotations were determined with a Hartnack HA-4001 polarimeter.

I.r. spectra were obtained by the KBr technique, using a Perkin-Elmer 1420 spectrophotometer.

Methylation analysis. — A modification[26] of the Hakomori methylation method[24] was used except that treatment with the base was for 16 h. The polysaccharide (10–15 mg) was dried overnight at 50° *in vacuo* and then methylated. Methylated fractions, which showed negligible i.r. absorption for hydroxyl, were hydrolysed sequentially at 100° with aqueous 90% formic acid (2 h) and 0.25M H_2SO_4 (16 h), and the products were reduced with $NaBD_4$, then acetylated[25,27], and subjected[25,28] to g.l.c. on 3% OV-225 at 180° (for the retention times) or with a temperature programme (for peak areas), and on 3% ECNSS-M at 180°. For quantitative determinations, the molar response factors recommended by Sweet *et al.*[29] were used. G.l.c.–e.i.-m.s. was performed on a VG 12-250 automatic mass spectrometer coupled to a Konik-2000 C series gas chromatograph, operated in the split mode, using an SE-30 capillary column (20 m × 0.22 m) at 190° or from 160 to

TABLE I

COMPOSITION[a] (%) OF THE POLYSACCHARIDES EXTRACTED WITH M SODIUM HYDROXIDE FROM *Penicillium erythromellis* CELL-WALL MATERIAL

Fraction	*Total "anhydro-hexose"*	*Neutral Sugars*										*Protein*
		Ara		*Xyl*		*Man*		*Gal*		*Glc*		
		b	*c*	*b*	*c*	*b*	*c*	*b*	*c*	*b*	*c*	
F1	74	0.1	tr.	0.8	2.0	1.8	4.5	36	29	6.5	25	16
F1S	73	0.2	0.9	0.2	0.4	2.2	5.3	39	33	6.4	15	18
F1P	66	tr.	0.5	2.3	3.7	0.3	1.9	6.9	6.4	3.1	49	9

[a]Mean value of duplicate determinations. [b]Obtained after hydrolysis with 0.05M H_2SO_4 for 5 h at 100°. [c]Obtained after Saeman hydrolysis for 2.5 h.

TABLE II

COMPOSITION[a] (%) OF THE FRACTIONS OBTAINED FROM F1S ON PURIFICATION THROUGH DEAE-SEPHAROSE

Fraction	*Recovery from column[d] (%)*	*Total "anhydro-hexose"*	*Neutral Sugars*										*Protein*
			Ara		*Xyl*		*Man*		*Gal*		*Glc*		
			b	*c*	*b*	*c*	*b*	*c*	*b*	*c*	*b*	*c*	
A	9.0	64	0.5	0.4	0.4	0.6	0.3	0.2	0.3	1.1	7.5	41	7.3
B	5.9	44	tr.	0.6	0.1	1.5	0.5	1.1	0.9	0.7	4.8	25	15
C	14.1	49	tr.	0.1	0.1	0.1	1.7	2.1	25	17	4.4	10	20
D	41.9	86	0.1	tr.	0.1	0.0	2.1	4.8	50	47	7.3	13	4.2
E	12.2	61	1.0	0.0	0.0	0.0	0.9	4.9	36	31	3.2	6.6	14

[a]Mean value of duplicate determinations. [b]Obtained after hydrolysis with 0.05M H_2SO_4 for 5 h at 100°. [c]Obtained after Saeman hydrolysis for 2.5 h. [d]Expressed as dry-weight percent of the starting material.

190° at 4°/min.

RESULTS

Monosaccharide composition of F1, F1S, and F1P.. — F1 represented 14.4% of the total cell wall, and F1 and F1S were composed mainly of galactose, glucose, mannose, and protein (Table I). F1S represented 68% of F1. F1P was mainly composed of glucose, galactose, xylose, mannose, and protein, and represented 20% of F1. Galactose was in the furanoid form in these fractions, since it was released after hydrolysis with 0.05M H_2SO_4 for 5 h at 100°. There was <1% of chitin in each of the three fractions.

Isolation of polysaccharides from F1S. — The elution pattern for chromatography on DEAE-Sepharose is shown in Fig. 1. Two main peaks (*A*, 9%; *D*, 41.9%) and several other fractions (*B*, *C*, and *E*) were analysed (Table II). Each fraction contained carbohydrate (44–86%) and protein (4.2–20%). The main neutral sugar in *A* and *B* was glucose. In *C–E*, the main neutral sugars were galactose, glucose, and mannose; *D* had the highest content (50%) of galactose and the lowest content (4.2%) of protein. Galactose was in the furanoid form in each fraction, since it was completely released on hydrolysis with 0.05M H_2SO_4 for 5 h at 100°. In *D* and *E*, the material resistant to this hydrolysis amounted to 37.6% and 46.7%, respectively, and ~9% of mannose, 4% of glucose, and traces of galactose were released after Sae-

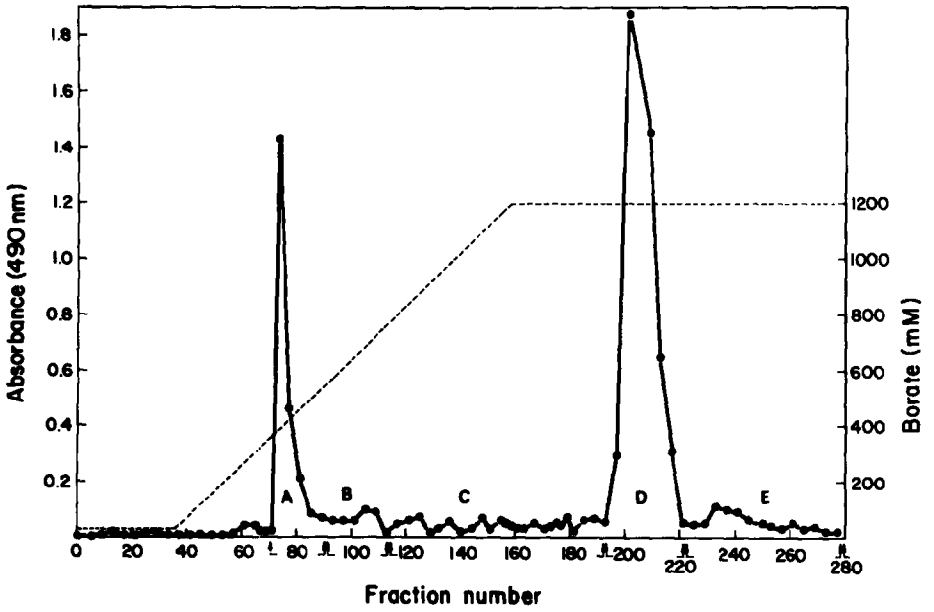

Fig. 1. Column chromatography of F1S on DEAE-Sepharose: —●—, total carbohydrate; ----, solvent gradient. For details, see text.

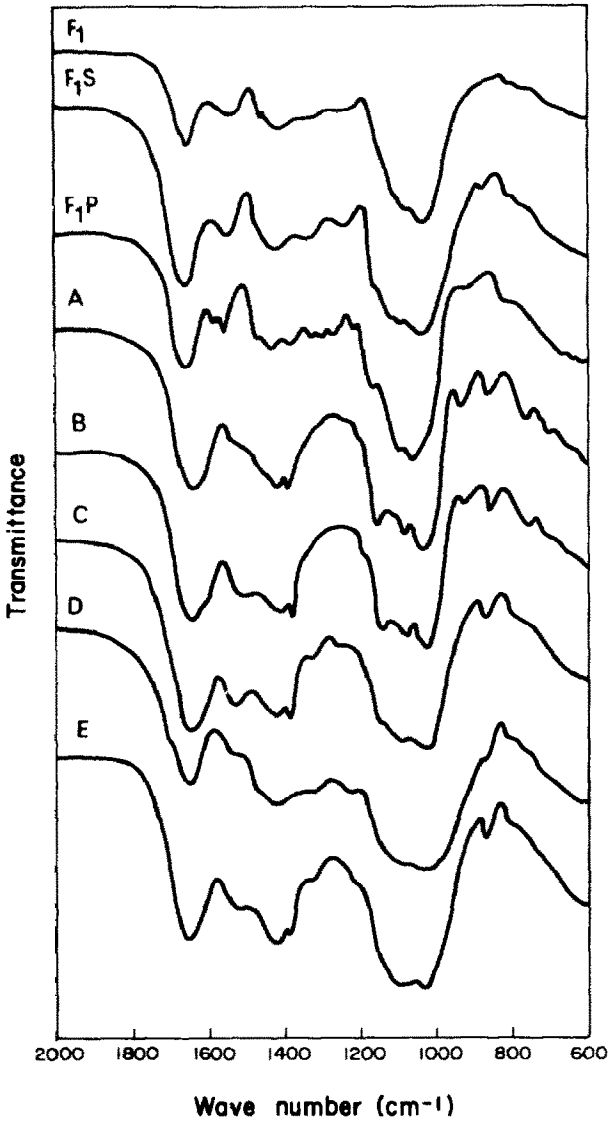

Fig. 2. I.r. spectra of *P. erythromellis* cell-wall polysaccharides: F1, extracted with M NaOH, neutralised, and dialysed; F1S, supernatant of the dialysate; F1P, sediment; A–E were obtained from F1S (see Fig. 1).

man hydrolysis for 2.5 h. Glucose and mannose were much more resistant than galactose to release in the acid hydrolysis, and the carbohydrate values obtained (anthrone method) were always higher than those obtained for neutral sugars after Saeman hydrolysis. None of the fractions contained phosphate ion.

I.r. spectra. — Each fraction showed absorptions at 1650 and 1560 cm^{-1} for protein (Fig. 2). These bands were smaller for *A* and *D*, in which protein content was lower. The bands at 805 and 870 cm^{-1} for F1, F1S, and *C–E* could be attributed

to galactofuranosyl residues[30], and they masked the absorption band of β-linked polysaccharides. F1P had absorption at 900 cm^{-1} characteristic of β-linked polysaccharides. *A* and *B* had i.r. spectra similar to that of glycogen[31], with absorptions at 760, 850, and 930 cm^{-1}. Absorption at 850 cm^{-1} is characteristic[32] of polysaccharides containing α sugars, and bands at 930 and 760 cm^{-1} are associated with (1→4)-linked α-D-glucans.

Optical rotation. — The following $[\alpha]_D^{20}$ values were obtained: F1P, −37.5° (*c* 0.2, M sodium hydroxide); and *D*, −25° (*c* 0.4, water); consistent with β configurations of the units.

Methylation analysis. — The results for the major fractions (*A*, *D*, and *E*) obtained from F1S are shown in Table III. *A* gave mainly acetylated 2,3,6-Me_3- and 2,3-Me_2-glucitol, suggesting that the polysaccharide contained a (1→4)-linked glucan backbone with branch points at positions 6. Glucopyranose and galactofuranose occurred as terminal residues. The formation of 2,4,6-Me_3-glucitol suggested (1→3)-linked glucose (3%). The total amount (~9%) of terminal residues agreed with the proportion of branch points in the glucan backbone (2,3-Me_2-glucitol, ~12%).

D and *E* gave mainly 2,3,6-Me_3-glucitol and 3,5,6-Me_3-galactitol, suggesting the presence of (1→4)-linked glucopyranose and (1→2)-linked galactofuranose in the polysaccharide backbone. The formation of 3,5-Me_2-galactitol indicated branch points at position 6 of the galactofuranose. Galactofuranose and glucopyranose were also found as terminal residues. In these fractions, the proportion of terminal residues was lower than the proportion of branch points.

DISCUSSION

The polysaccharidic material ($F1^{7,10}$) extracted from *P. erythromellis* cell wall with M alkali, when neutralised and dialysed, yielded a precipitate (F1P) and a supernatant solution from which a galactofuranose-rich material was obtained (F1S). These fractions represented 2.9% and 9.8% of the cell wall, respectively.

F1P was a β-glucan containing minor proportions of galactose, xylose, and mannose, the structural features of which were not determined.

Chromatography of F1S on DEAE-Sepharose yielded three peaks (*A*, *D*, and *E*) with a higher carbohydrate content. *A* was a (1→4)-α-D-glucan with branch points at positions 6 and has not been described hitherto as a constituent of *Penicillium* cell wall. Troy and Koffler[3] reported the presence of a (1→3)-β-D-glucan in *P. chrysogenum* cell walls and Matsunaga *et al.*[14], on the basis of ^{13}C-n.m.r. data and susceptibility to amylase, reported a (1→4)-α-D-glucan probably located in the cytoplasm of *P. ochro-chloron*.

D (4.1% of the cell wall) was a glucogalactan with (1→4)-linked glucopyranosyl (34.5%) and (1→2)-linked galactofuranosyl residues (43.2%) in the backbone. The same linkages were found in *E*, but in different proportions. (1→2)-Linked β-galactofuranosyl residues have been described only in the cell-wall polysaccharides of *P. ochro-chloron*, in which this fraction was 6.2% of the total cell wall[14]. *P.*

TABLE III

G.L.C.–M.S. DATA FOR THE METHYLATED ALDITOL ACETATES FROM F1S FRACTIONS[a]

Alditol	T[b]	*Relative mol*(%)			*Major mass spectrum fragments* (m/z)	*Deduced linkage*
		A	D	E		
2,3,5,6-Me_4-Gal[c]	1.07	3.4	1.5	3.9	45, 59, 89, 101, 102, 118, 205	Gal*f*-(1→
3,5,6-Me_3-Gal	1.98	—	25.8	23.2	45, 59, 88, 89, 101, 130, 145, 190, 205, 306	→2)-Gal*f*-(1→
Me_3-Gal	2.79	—	3.6	6.0	87, 101, 102, 117, 118, 129, 162, 189	
3,5-Me_2-Gal	5.11	—	17.4	12.0	88, 101, 117, 130, 173, 190, 233	→2,6)-Gal*f*-(1→
2,6-Me_2-Gal	3.14	9.5	—	—	45, 87, 118, 129	→3,4)-Gal*p*-(1→
						→3,5)-Gal*f*-(1→
2,3,4,6-Me_4-Glc	1.00	5.8	9.7	7.8	45, 87, 101, 102, 118, 129, 145, 161, 162, 205	Glc*p*-(1→
2,3,6-Me_3-Glc	2.32	65.8	34.5	35.8	45, 87, 99, 102, 113, 118, 129, 162, 172, 233	→4)-Glc*p*-(1→
2,4,6-Me_3-Glc	1.74	3.0	0.3	—	87, 101, 118, 129, 161, 234	→3)-Glc*p*-(1→
2,3-Me_2-Glc	4.21	12.2	—	—	85, 102, 118, 261	→4,6)-Glc*p*-(1→
3,4-Me_2-Hex	4.37	—	7.0	11.4	87, 88, 129, 130, 189, 190	→2,6)-Man*p*-(1→

[a]Hydrolysis with 0.25M H_2SO_4 for 16 h at 100°. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on OV-225 at 180°. [c]2,3,5,6-Me_4-Gal = 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylgalactitol, etc.

ochro-chloron cell-wall glucogalactans have two types of β-galactofuranosyl residues, one of which has (1→2) linkages and is composed of 77.3% of galactose and 22.7% of glucose. Both β-galactofuranans are minor cell-wall components, as in *P. erythromellis*. Gander and Fang[12] reported a polymer composed primarily of galactofuranosyl and glucosyl residues in *P. charlesii* cell walls. The galactofuranosyl residues contribute to the antigenic specificity of *Penicillium* species[15].

B and *C* were, most probably, polysaccharide-protein complexes, with different proportions of glucose and galactose. An extracellular peptidophosphogalactomannan has been isolated from *P. charlesii*[13].

These results show that the alkali-soluble fraction obtained from *P. erythromellis* cell walls contains a mixture of heteropolysaccharides. The polysaccharides containing galactofuranose are not present in other species of *Penicillium*, in which only an α-D-glucan has been found. These differences in cell-wall composition indicated that there are at least two types of cell wall within this genus, as has been reported by Leal *et al.*[7]. These results also show the complexity of *Penicillium* cell walls.

The study of the fine structure of these minor polysaccharide components of *Penicillium* cell wall could provide data of value in the chemotaxonomy of *Penicillium* and related genera.

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REFERENCES

1 D. A. APPLEGARTH, *Arch. Biochem. Biophys.*, 120 (1967) 471–478.
2 P. B. HAMILTON AND S. G. KNIGHT, *Arch. Biochem. Biophys.*, 99 (1962) 282–287.
3 F. A. TROY AND H. KOFFLER, *J. Biol. Chem.*, 244 (1969) 5563–5576.
4 D. A. APPLEGARTH AND G. BOZOIAN, *Can. J. Microbiol.*, 14 (1968) 489–490.
5 V. GRISARO, N. SHARON, AND R. BARKAI-GOLAN, *J. Gen. Microbiol.*, 54 (1968) 145–150.
6 P. D. UNGER AND A. W. HAYES, *J. Gen. Microbiol*, 91 (1975) 201–206.
7 J. A. LEAL, A. MOYA, B. GOMEZ-MIRANDA, P. RUPEREZ, AND C. GUERRERO, IN C. NOMBELA (Ed.), *FEMS Symposium Proceedings, 27*, Elsevier, Amsterdam, 1984, pp. 149–155.
8 B. GOMEZ-MIRANDA AND J. A. LEAL, *Microbiologia*, 1 (1985) 67–75.
9 B. GOMEZ-MIRANDA, C. GUERRERO, AND J. A. LEAL, *Exp. Mycol*, 8 (1984) 298–303.
10 P. RUPEREZ AND J. A. LEAL, *Trans. Br. Mycol. Soc.*, 86 (1986) 279–285.
11 J. E. GANDER, *Annu. Rev. Microbiol.*, 28 (1984) 103–119.
12 J. E. GANDER AND F. FANG, *Biochem. Biophys. Res. Commun.*, 71 (1976) 719–725.
13 J. E. GANDER, N. H. JENTOFT, L. R. DREWES, AND P. D. RICK, *J. Biol. Chem.*, 249 (1974) 2063–2072.
14 T. MATSUNAGA, A. OKUBO, M. FUKAMI, S. YAMAZAKI, AND S. TODA, *Biochem. Biophys. Res. Commun.*, 102 (1981) 524–530.
15 J. F. PRESTON, E. LAPIS, AND J. E. GANDER, *Can. J. Microbiol.*, 16 (1970) 687–694.
16 P. RUPEREZ, B. GOMEZ-MIRANDA, AND J. A. LEAL, *Trans. Br. Mycol. Soc.*, 80 (1983) 313–318.

17 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
18 M. C. RAND, A. E. GREENBERG, AND M. J. TARAS, *Standard Methods for the Examination of Water and Wastewater*, 14th edn., WPCF, APHA, AWWA, 1975.
19 R. DREYWOOD, *Ind. Eng. Chem., Anal. Ed.*, 18 (1946) 499.
20 G. A. ADAMS, *Methods Carbohydr. Chem.*, 5 (1965) 269–276.
21 R. R. SELVENDRAN, J. F. MARCH, AND S. G. RING, *Anal. Biochem.*, 96 (1979) 282–292.
22 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
23 G. C. CHEN AND B. R. JOHNSON, *Appl. Env. Microbiol.*, 46 (1983) 13–16.
24 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
25 S. G. RING AND R. R. SELVENDRAN, *Phytochemistry*, 17 (1978) 745–752.
26 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LONNGREN, *Chem. Commun. Univ. Stockholm*, 8 (1976).
27 B. J. H. STEVENS AND R. R. SELVENDRAN, *J. Sci. Food Agric.*, 31 (1980) 1257–1267.
28 P. RUPEREZ, B. GOMEZ-MIRANDA, AND J. A. LEAL, *Can. J. Microbiol.*, 30 (1984) 1157–1162.
29 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225.
30 S. A. BARKER AND R. STEPHENS, *J. Chem. Soc.*, (1954) 4550–4555.
31 A. J. MICHELL AND G. SCURFIELD, *Aust. J. Biol. Sci.*, 23 (1970) 345–360.
32 S. A. BARKER, E. J. BOURNE, AND D. H. WHIFFEN, *Methods Biochem. Anal.*, 3 (1956) 213–245.