Production of soluble lignin-rich fragments (APPL) from wheat lignocellulose by *Streptomyces viridosporus* and their partial metabolism by natural bacterial isolates

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Summary

The actinomycete *Streptomyces viridosporus* attacks wheat lignocellulose releasing soluble lignin-rich fragments (APPL). Chemical analyses indicated that these APPL contain 20% less guaiacyl and 70% less glucose residues than the original substrate. In order to determine the effect of non-filamentous bacteria on APPL, natural isolates were selected in synthetic media containing APPL as sole carbon source. From a total of eighty cultures, two strains (*Pseudomonas* spp. B_{23} and E_{21}) and a consortium of two strains (*Enterobacter* sp. V_1 and *Pseudomonas* sp. V_2) were selected for further studies. Strains *Pseudomonas fluorescens* biovar I and *Pseudomonas acidovorans* D_3, previously isolated on β-1 and β-O-4 lignin model compounds, respectively, were included for comparative purposes. Analysis of APPL recovered after bacterial growth indicated that *Pseudomonas* B_{23} attacked preferentially both guaiacyl and syringyl lignin units while barely affected their carbohydrate content. On the other hand, *Pseudomonas* E_{21} and the consortium metabolized sugar moieties without modifying the aromatic residues of APPL. *P. fluorescens* biovar I and *P. acidovorans* D_3, in spite of being able to cleave lignin linkages on dimeric model compounds, exhibited very limited growth on APPL. The

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former only partially degraded guaiacyl residues, while the latter did not produce detectable changes on APPL structure.

Lignocellulose; APPL; Lignin oligomer; *Streptomyces viridosporus*; Pseudomonas

Introduction

Recent studies have established that several *Actinomycetes* attack grass lignocelluloses leading to partial solubilisation of the substrate rather than to its mineralization (McCarthy, 1987). The material released to the culture media has been shown to consist mainly of a mixture of heterogeneous polymers highly enriched in lignin residues that are insoluble at low pH. Due to these properties, the solubilized fragments have been called "APPL", for acid precipitable polymeric lignin (Crawford et al., 1983). The lignocellulolytic processes have been investigated using *Thermomonospora mesophila* (McCarthy et al., 1986) and a few species of *Streptomyces* (Borgmeyer and Crawford, 1985; Mason et al., 1988). Characteristics of APPL differ according to the culture conditions, the harvesting procedure and the strains that produce them. For example, APPL collected by acid precipitation present higher average molecular weight than those obtained by lyophilization (Crawford et al., 1983). The strain *S. viridosporus* releases APPL in both solid and liquid processes, whereas *S. badius* solubilizes lignocellulose in solid media only (Borgmeyer and Crawford, 1985). However, in spite of individual variations, APPL share some common properties: a molecular weight of > 5000 Da, a carbohydrate moiety (7–20%) composed mainly of hemicelluloses and a nitrogen content of about 4% (McCarthy, 1987).

Two lines of evidence indicate that APPL themselves are slowly degraded by *Actinomycetes*. Addition of radiolabelled soluble polymer to fresh cultures of *T. mesophila* resulted in only 2.0–2.5% further mineralization (McCarthy and Broda, 1984). On the other hand, incubation of APPL produced by *S. viridosporus* with other lignocellulose-degrading *Streptomyces* strains led to limited additional degradation (Pometto and Crawford, 1986). The ligninolytic fungus *Phanerochaete chrysosporium* does not degrade APPL extensively either (Pometto and Crawford, 1986).

Since the catabolic action of the above microorganisms does not account for the complete degradation of APPL, the possibility exists that non-filamentous bacteria might play a role in this process. For this reason, we decided to examine the effect of other bacterial taxa on this metabolic intermediate. APPL, being soluble in water, represent a suitable high molecular weight natural substrate for lignin biodegradation studies.

Materials and Methods

Chemicals

Anisoin (4,4′-dimethoxybenzoin), 4,4′biphenol, sodium borohydride and caffeic, cinnamic, coumaric, ferulic, *p*-hydroxybenzoic, protocatechuic, syringic and vanillic acids were purchased from Aldrich Chemical Co. Ethanethiol, benzophenone, boron trifluoride etherate, tetracosane, glucose, galactose, maltose and acetic anhydride were from Merck, Darmstadt, FRG. Inositol was obtained from Matheson, Coleman & Bell, NJ. Arabinose, fructose, mannose, ribose and xylose were from Fluka A.G. Buchs SG, Switzerland. Galactomannan (locust bean gum) and microcrystalline cellulose (Sigma-cell) were from Sigma Chemical Co. Arabino-galactan (reinst, 95%) was obtained from Serva GMBH & Co., Heidelberg, F.R.G. Xylan (ex larch sawdust) was purchased from Koch-Light Lab, Coinbrook, Bucks, England. Hydroxyethyl cellulose was kindly donated by Dr. J. Sapag (U. de Chile, Santiago, Chile). Guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether were synthesized according to procedures previously described (Landucci et al., 1981; Adler et al., 1952). Hydroxymethyldesoxyanisoin (HMDA, 3-hydroxy-1,2-bis(4′-methoxyphenyl)-propanone) was obtained as described (Kirk and Nakatsubo, 1983).

Preparation of lignocellulose

Ground wheat straw (0.25–2.0 mm) of whole plants was subjected to successive toluene-ethanol, ethanol and water extractions and then dried at 50 °C as described (Crawford and Crawford, 1976).

Culture conditions for S. viridosporus

Stock slants of the strain, which was a generous gift of Dr. D.L. Crawford (Navarre, MN), were kept on Luria medium at 4 °C and routinely transferred to fresh slants every 2–3 weeks.

Preparation of APPL

APPL from wheat lignocellulose were produced based on the protocol previously described by Crawford et al. (1983). Briefly, inocula were prepared by adding 2.5 ml of a turbid solution of spores ($OD_{600\ nm}$ 0.3) to each flask containing 22.5 ml of a salt medium (Crawford, 1978) supplemented with 0.3% of yeast extract and incubated with agitation at 37 °C for 48 h. Cultures with full grown mycelia were transferred to bottles previously autoclaved with 2.5 g of dampened wheat lignocellulose. Each vessel was carefully rolled to distribute both the lignocellulose and the suspension of bacteria evenly on its inside surface. *S. viridosporus* was incubated on this solid substrate at 37 °C in a water bath for the time intervals indicated in the text. Then, 125 ml of water were added to each bottle, the suspension autoclaved at 100 °C for 1 h, filtered and dialyzed against water. The product was recovered by lyophilization.

Isolation, culture conditions and identification of non-filamentous bacteria

Soil samples used as a source of microorganisms were added to cultures (González et al., 1986) containing 2.5 mg ml^{-1} of APPL harvested after two weeks as sole carbon and energy source. In order to prepare sterile stock solutions (5.0 mg ml^{-1} in distilled water), APPL were first filtered through Whatman No. 1 paper and then filter-sterilized through membranes of 45 μm of pore diameter. After four transfers in this enrichment culture and plating on Luria medium, a total of 80 isolates were examined. The three cultures which grew best in minimal medium containing APPL (OD_{600nm}) between 0.25 and 0.55) were selected for further studies.

The metabolic versatility of the selected bacterial strains was assayed by replacing APPL in the same synthetic medium by simple aromatic acids, dimeric lignin model compounds or carbohydrates. Final concentrations of single ring aromatic compounds and dimeric lignin model structures were 5.0 mM and 1.0 mg ml^{-1}, respectively. Simple sugars were usually tested at a final concentration of 1.0% and complex sugars were assayed at 0.5%.

Three of the selected strains (B_{23}, E_{21} and V_2) were identified as *Pseudomonas* based on the following evidence: Gram-negative motile rods that produce yellow pigment are oxidase positive and do not degrade glucose. Strain V_1 did not conform to the above pattern: it is a Gram-negative rod possessing peritrichous flagella, does no produce yellow pigments, it is oxidase negative and degrades glucose. This isolate was therefore ascribed to the genus *Enterobacter*. Additional tests performed with all strains included ability to grow either on asparagine, in the absence of growth factors, proliferation at 5 °C and capability to carry out hydrolysis of starch and gelatin.

All strains were maintained at 4 °C in salt medium containing 0.4% agar and 1.5 mg ml^{-1} of APPL.

Analytical procedures

Gel permeation chromatography. Molecular weight distribution of APPL was determined by HPLC using a Perkin Elmer Series 2 chromatograph equipped with a Shodex Ion Pak S-804/S column and a Perkin Elmer UV-detector. Elution of APPL was monitored at 280 nm. Samples were dissolved in and eluted with a solution containing 0.1 M NaOH and 0.05 M LiCl and the flow rate was adjusted to 1.0 ml min^{-1}. For calibration, a series of pullulans were used and their retention times recorded with a refractive index detector. The molecular weight distribution of APPL was also analyzed by filtration in a Sephadex G-50 column (70 cm × 1.1 cm^2) equilibrated with a solution containing 0.1 M NaOH and 0.1 M LiCl. The flow rate was adjusted to 10 ml h^{-1} and aliquots of 1.0 ml were collected.

Spectrophotometric measurements. UV spectra of culture supernatants containing APPL were registered in a Perkin Elmer Lambda 3B spectrophotometer. Emission or fluorescence spectra of solutions containing 0.08 mg ml^{-1} of APPL were determined with a Perkin Elmer 204-S fluorescence spectrophotometer attached to a

Xenon Perkin Elmer 150 power supply. Solutions were excited using wavelengths from 270–285 nm and the resulting emission spectra recorded in the range of 275 to 550 nm.

Analysis of lignin. For APPL analyses after incubation with bacteria, cells were sedimented and the supernatants freeze-dried before subjecting the substrate to the procedures described below. Thioacidolyses of lignin in lignocellulose and APPL were performed as reported (Lapierre et al., 1985); 3.0 – 20 mg of lignin sample were placed in a tube with a teflon-lined screw cap, together with 10 ml of dioxane-ethanethiol (9 : 1), 0.2 M BF_3 etherate, under nitrogen at 100 °C (oil bath) for 4 h with occasional shaking. Tubes were then cooled in ice and the reaction mixture was thereafter diluted with 10 ml of distilled water, adjusted to pH 3.0–4.0 with 0.4 M aqueous $NaHCO_3$ and extracted with CH_2Cl_2 (3×25 ml). The combined organic extracts, to which the gas chromatography (GC) internal standard (0.2 – 0.5 mg tetracosane) was added, were dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. The oily residue was redissolved in 0.5 – 1.0 ml CH_2Cl_2. The main hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomeric products were identified by gas chromatography-mass spectrometry (GC-MS), as their trimethylsilylated (TMSi) derivatives, and quantified by GC as previously described (Lapierre et al., 1986a,b).

Analysis of carbohydrates. Determination of glucose, xylose, mannose, galactose and arabinose contained in lignocellulose and APPL was performed in the form of their alditol acetate derivatives (see anonymous reference "Provisional Method"). The original protocol was scaled down and set up with some modifications: samples of 50 mg of wheat lignocellulose (60 mesh) or APPL were hydrolyzed in 0.75 ml of 72% H_2SO_4 (v/v) for 1 h at 30 °C, swirling occasionally. After this step, samples were diluted with 21 ml of water, autoclaved for 30 min at 120 °C and cooled to room temperature. After addition of the inositol standard solution, samples were neutralized with 1.05 g $CaCO_3$, centrifuged and the pellet carefully washed until completing 50 ml of clear supernatant. The sugars contained in this solution were converted to the corresponding alditols adding 80 mg of $NaBH_4$. The excess of reductant was eliminated after 2 h with glacial acetic acid. The mixture was then evaporated, washed with methanol and dried. Alditols were acetylated at 55 °C with 7.5 ml of acetic anhydride and 0.5 ml of concentrated H_2SO_4. Samples were then cooled to 4 °C, diluted with 30 ml of water and 40 g of ice and extracted three times with CH_2Cl_2. They were then evaporated and quantitatively resuspended in 1.0 ml of CH_2Cl_2. GC analyses of 1.0 µl aliquots were done with a Tracor 560 chromatograph equipped with a 10 ft cyanosilicone column (3.0% SP2330 on chromosorb WHP) attached to a flame ionization detector. The carrier gas was nitrogen and the column was maintained at 250 °C.

Determination of total and reducing sugars. Degradation of celluloses and hemicelluloses by non-filamentous bacterial strains was monitored by measuring the contents of both total and reducing sugars. Total carbohydrate content was estimated in duplicate samples by the colorimetric orcinol method (Rimington, 1940).

A calibration curve of glucose in the range of 0.5 – 20.0 mg ml^{-1} was utilized. Reducing sugars were determined using the Nelson-Somogyi method (Nelson, 1944).

Results

Characterization of the acid-precipitable product released by S. viridosporus *from wheat lignocellulose*

S. viridosporus was incubated in solid media of sterile wheat lignocellulose and the solubilization of the substrate was followed over time. After two weeks of incubation, conversion to APPL was approximately 5.0% (w/w) of the initial lignocellulose in all experiments, a value that remained constant after one month of additional incubation. The MW distribution of the collected polymer was determined by gel permeation HPLC using a Shodex S-804/S column. The elution profile showed a major peak ranging in size between 5,000 and 20,000 Da and also a small fraction of higher MW (> 50,000 Da) (Fig. 1). Heterogeneity in MW of APPL was confirmed by filtration through a Sephadex G-50 column. A significant fraction eluted as a sharp peak in the excluded volume, indicating an apparent MW of at least 20,000 Da, whereas about 30–40% of the material eluted in a broad band of lower MW (Fig. 2). The above elution profiles remained unaltered starting from the 7th day of growth of the filamentous bacterium on wheat lignocellulose. On the other hand, sterilization of APPL by millipore filtration (45 μm) led to a loss of part of the high MW fraction of the substrate (Fig. 2). The molecular size distribution

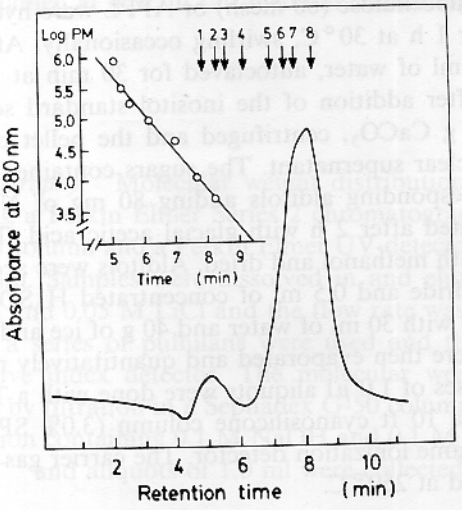

Fig. 1. HPLC elution profile of APPL harvested after incubation of *S. viridosporus* on wheat lignocellulose for 2–6 weeks. Samples were eluted with an aqueous solution of 0.1 M LiCl and 0.05 M NaCl at a flow rate of 1 ml min^{-1}. The column (Shodex Ion Pak 804/S was calibrated with standard pullulans of known MW: (1) 75.8×10^4 Da; (2) 33.8×10^4 Da; (3) 19.4×10^4 Da; (4) 9.54×10^4 Da; (5) 4.67×10^4 Da; (6) 2.08×10^4 Da and (7) 0.53×10^4 Da.

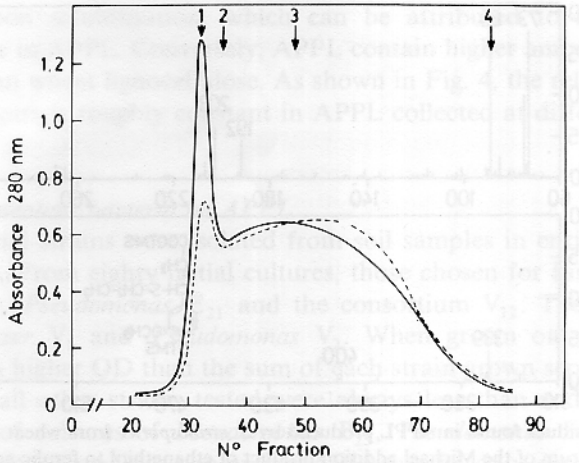

Fig. 2. Sephadex G-50 permeation of APPL before (———) and after (------) sterilization of 1.0 ml aliquots of a 5 mg ml^{-1} solution through a 45 μm membrane filter. Arrows indicate elution volumes of (1) Blue dextran, (2) chymotrypsinogen A (MW 24 kDa), (3) lysozyme (MW 14 kDa) and (4) coumaric acid (MW 164 Da).

described for APPL, however, may not necessarily represent absolute molecular weight values, since gel permeation chromatography can be affected by interaction between APPL components and the column matrix.

Analysis of the lignin fraction of lignocellulose and APPL was performed by thioacidolysis. This technique selectively cleaves β-O-4 linkages and can be used to quantitate the relative content of hydroxyphenyl (H), guaiacyl (G) and syringyl (S) residues of uncondensed lignin (Lapierre et al., 1986a; Rolando et al., 1989). When APPL harvested after two weeks of incubation and lignocellulose were compared, a lower G content in the former was detected (Table 1). This modification produces a shift in the S/G ratio, reflecting a significant change in the structural composition of uncondensed lignin. Furthermore, GC-MS analysis of APPL showed a peak corresponding to the Michael addition product of ethanethiol to the olefinic double bond of ferulic acid (Fig. 3). In the case of wheat lignocellulose, this peak was

TABLE 1

ANALYSIS BY THIOACIDOLYSIS OF AROMATIC RESIDUES FROM WHEAT LIGNOCELLULOSE AND APPL

Sample	H%	G%	S%	S/G
Lignocellulose	0.13 (0.015)	1.77 (0.140)	1.66 (0.130)	0.94 (0.00)
APPL	0.14 (0.005)	1.44 (0.015)	1.58 (0.035)	1.097 (0.01)
P value	NS	$P < 0.02$	NS	$P < 0.001$

Results are expressed as percentage over total weight of sample (w/w). Samples were analyzed in triplicate. Standard deviations are indicated in parentheses. H = *p*-hydroxyphenyl; G = guaiacyl and S = syringyl residues. Values of *P* were determined for lignocellulose and APPL according to Student's *t*-test. NS = not significant.

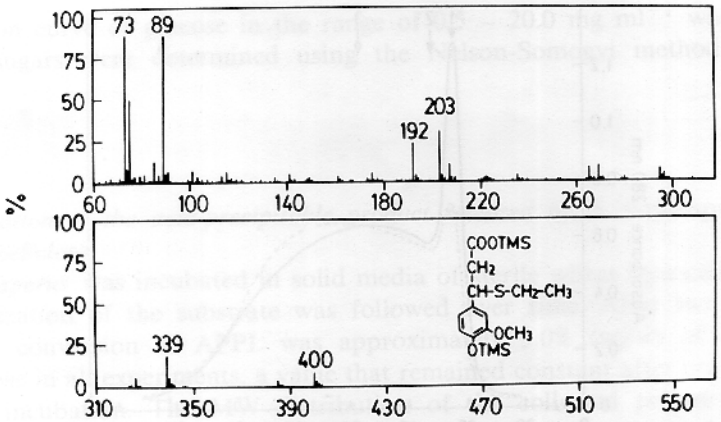

Fig. 3. Ferulic acid residues found in APPL produced by *S. viridosporus* from wheat lignocellulose. Mass spectrum of the Michael addition product of ethanethiol to ferulic acid.

hardly detectable, which suggests that the wheat APPL fraction is enriched in ferulic units, compared to the whole cell wall. A low percentage of H was also found in both samples. This residue, commonly found in grass lignins (Higuchi, 1985), as well as the syringyl groups, remained unchanged during the solubilisation process.

Quantitative analysis of carbohydrates were also performed, following the procedure described in Materials and Methods. Fig. 4 summarizes the modifications found in the sugar composition of lignocellulose and APPL harvested at different incubation times. An overall decrease in the sugar content from 30 to about 20%

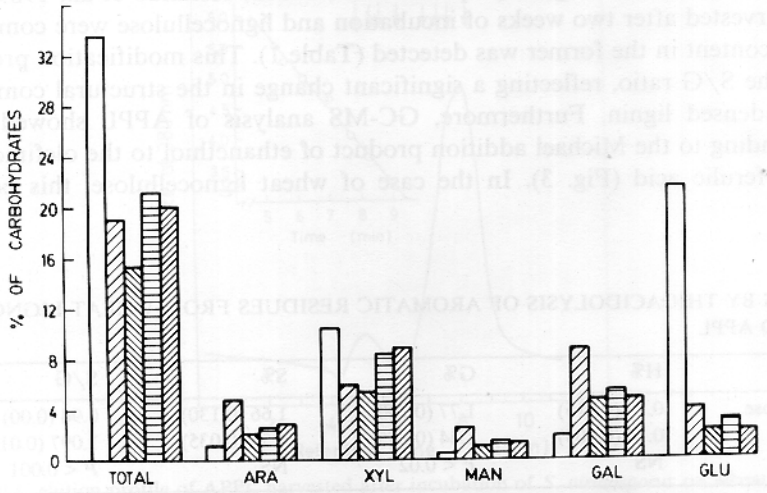

Fig. 4. Carbohydrate analysis of wheat lignocellulose and APPL derived from it by the action of *S. viridosporus*. Total sugars and arabinose (ARA), xylose (XYL), mannose (MAN), galactose (GAL) and glucose (GLU) were measured. From left to right values for lignocellulose, and APPL collected after 0 d, 4 d, 8 d and 2–6 weeks are indicated.

was observed upon solubilisation, which can be attributed to a sharply lower content of glucose in APPL. Conversely, APPL contain higher amounts of galactose and arabinose than wheat lignocellulose. As shown in Fig. 4, the relative proportion of the various sugars is roughly constant in APPL collected at different incubation times.

Effect of non-filamentous bacteria on APPL

Natural bacterial strains were isolated from soil samples in enrichment cultures containing APPL. From eighty initial cultures, those chosen for further study were *Pseudomonas* B_{23}, *Pseudomonas* E_{21} and the consortium V_{12}. The latter included strains *Enterobacter* V_1 and *Pseudomonas* V_2. When grown on APPL, the consortium reached a higher OD than the sum of each strain grown separately, whereas combinations of all other strains tested were always less than additive. Maximum optical densities of cultures of *Pseudomonas* B_{23}, *Pseudomonas* E_{21} and the consortium V_{12} in culture medium containing APPL as the only carbon source were 0.25, 0.37 and 0.55, respectively. Growth curves indicated that all three cultures reached stationary phase in less than 50 h.

The action of these bacteria on APPL was analyzed by chromatographic, spectrophotometric and chemical methods. For comparative purposes, two additional strains were included in this study. These were *P. fluorescens* biovar I and *P. acidovorans* D_3, which are known to cleave intermonomeric linkages in dimeric lignin model compounds. The former cleaves both the β-1 and β-O-4 bonds (González and Vicuña, 1989 and unpublished observations), whereas the latter cleaves β-O-4 linkages of both phenolic and non-phenolic structures (Vicuña et al., 1987). Growth of these two strains on APPL was however poor, their maximum optical densities not surpassing a value of 0.1 even after a prolonged incubation time.

Cultures of all selected strains were grown at 30 °C for 4 d in salt medium containing 2.5 mg ml^{-1} of pre-filtered APPL. Cells were then separated by centrifugation and samples of the supernatants tested for possible modifications of the substrate. Spectrophotometric analyses of aliquots of the five cultures examined revealed no difference with respect to uninoculated controls. Emission spectra of the same samples did not show any change after 2 or 4 d of incubation when compared to sterile incubated controls. Chromatographic analyses using either Sephadex G-50 or Shodex Ion-Pak S-804/S gel filtration exhibited no significant modification of the elution profile of the substrate.

Chemical analyses of these samples were also performed. Thioacidolysis yielded the results summarized in Table 2. Strain *Pseudomonas* B_{23} produced a significant decrease of both the G and S residues recovered from the reaction mixture when compared to the sterile control. *P. fluorescens* biovar I also attacked the aromatic fraction of APPL lowering the amount of G residues recovered, thereby affecting the S/G ratio significantly. All other cultures did not alter the aromatic component of the substrate as measured by this technique. Values of G and S of the sterile control are lower than those shown in Table 1. This difference was attributed to the high salt content of the samples after lyophilization of culture medium. Samples

TABLE 2

ANALYSIS BY THIOACIDOLYSIS OF THE AROMATIC FRACTION OF FILTER-STERILIZED APPL AFTER INCUBATION WITH BACTERIAL STRAINS

Sample	G%	S%	S/G	N [a]
Sterile control	0.688 (0.115)	0.649 (0.085)	0.945 (0.048)	4
Pseudomonas B23	0.515 (0.055) [a]	0.497 (0.055) [b]	0.960 (0.008)	4
Pseudomonas E21	0.568 (0.177)	0.649 (0.177)	0.957 (0.023)	3
Consortium V12	0.656 (0.122)	0.642 (0.138)	0.973 (0.035)	4
P. fluorescens	0.538 (0.032) [a]	0.564 (0.021)	1.045 (0.031) [c]	4
P. acidovorans D3	0.577 (0.000)	0.591 (0.044)	1.020 (0.071)	2

Results are expressed as percentage over total weight of samples. The number of determinations is indicated. Standard deviations are in parentheses. P values were obtained comparing each culture with the sterile incubated control according to Student's t-test. [a] $P < 0.05$; [b] $P < 0.025$; [c] $P < 0.02$.

were not dialyzed before subjecting to the above procedures in order to avoid loss of low MW material. Determination of sugars as their alditol acetates is shown in Fig. 5. Only *Pseudomonas* E_{21} and the consortium V_{12} produced an important decrease in the total carbohydrate content. The former consumed mainly mannose, whereas the mixed culture degraded xylose and mannose preferentially.

Metabolic characterization of bacterial strains isolated on APPL

The metabolic versatility of the selected strains was assessed by testing a range of

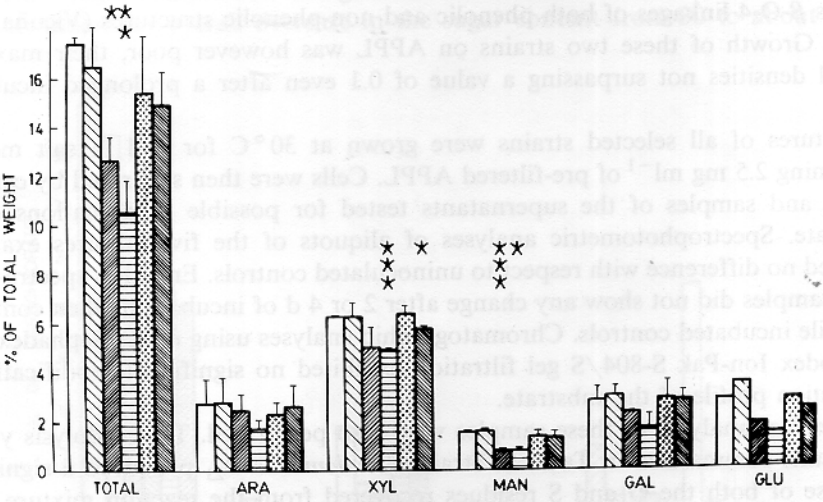

Fig. 5. Analysis of carbohydrates in filter-sterilized APPL after incubation with bacterial strains. Data from each culture is compared with the sterile incubated control. From left to right columns represent the sterile control, *Pseudomonas* B_{23}, *Pseudomonas* E_{21}, consortium V_{12}, *P. fluorescens* biovar I and *P. acidovorans* D_3. ARA = arabinose; XYL = xylose; MAN = mannose; GAL = galactose; GLU = glucose. Mean of two independent experiments and standard deviations are indicated. P values were obtained comparing each culture with the uninoculated control according to Student's t-test. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.005$. Values shown for glucose represent a single determination.

different compounds as sole carbon sources. Since APPL are a chemically heterogeneous substrate, both lignin-related compounds and carbohydrates were included in these analyses.

Single ring aromatic compounds tested were *p*-hydroxybenzoic, protocatechuic, vanillic, coumaric, ferulic, syringic and cinnamic acids. Four dimeric lignin model compounds were assayed as growth substrates as well: anisoin, HMDA, veratryl-glycerol-β-guaiacyl ether and 4,4′-biphenol. *Pseudomonas* B_{23} and E_{21} were able to grow on all the above single ring aromatic acids, with the sole exception of syringate. *Pseudomonas* V_2, which is one of the two strains of the consortium, did not metabolize ferulic, cinnamic or syringic acids, while the *Enterobacter* V_1 was able to grow only on protocatechuic acid. None of the strains selected on APPL degraded any of the four dimeric compounds when these were present as the sole carbon source in the culture medium, even when incubation were maintained during 10 or more days.

The ability of the selected strains to grow on simple carbohydrates was measured using glucose, galactose, mannose, maltose, fructose, arabinose, xylose and ribose. It was shown that *Pseudomonas* E_{21}, both strains of the consortium V_{12} and *P. fluorescens* biovar I metabolized all monosaccharides tested, with the exception of *Pseudomonas* E_{21}, which did not catabolize ribose. *Pseudomonas* B_{23} did not grow on any of the carbohydrates assayed, while *P. acidovorans* D_3 degraded only fructose. In turn, none of the strains was able to develop in culture medium containing either hydroxyethyl cellulose, microcrystalline cellulose, xylan, arabino-galactan or galacto-mannan. However, cells of *Pseudomonas* E_{21} grown on glucose could lower the total sugar content of xylan, galacto-mannan and arabino-galactan to 34.9, 55.0 and 49.6%, respectively.

Discussion

Although there have been several recent advances on the enzymology of APPL release (Deobald and Crawford, 1987; Mason et al., 1988; Ramachandra et al., 1987, 1988), the present work was focused on the production of this soluble oligomer for bacterial biodegradation studies.

Values for MW of APPL derived from wheat lignocellulose determined from chromatographic elution profiles are consistent with data obtained for APPL released by *S. viridosporus* from corn lignocellulose (Crawford et al., 1983) and by *T. mesophila* on wheat lignocellulose (McCarthy et al., 1986). As indicated in Methods, preparation of APPL involves a step of autoclaving after incubation of lignocellulose with the actinomycete. Analysis of non inoculated samples indicated that some soluble material had been released abiotically during the processes of incubation and autoclaving. This material, of much lighter colour than APPL, was subjected to the same chromatographic procedures, exhibiting a significantly reduced absorbance. It was not characterized further.

Thioacidolysis was selected for chemical characterization of lignin. The advantages of this method have been amply confirmed and are discussed elsewhere

(Lapierre et al., 1986a; Rolando et al., 1989). When compared to lignocellulose, APPL exhibit a decreased content of guaiacyl (G) monomers, whereas syringyl (S) and *p*-hydroxyphenyl (H) moieties remain unchanged. Several explanations are possible to account for the observed decrease in G residues. One possibility is that G residues may be preferentially attacked by a peroxidase recently described (Ramachandra et al., 1987, 1988). Alternatively, APPL may be released in a "passive" way and originate mainly from the secondary wall. If this was the case, the decrease of guaiacyl monomers would only reflect the heterogeneous nature of lignin structure within the different layers of lignocellulose. In situ studies of wheat straw lignin have shown that, in the uncondensed lignin moiety, 40% of G, but only 6.0% of S units, have a free phenolic group at C_4 and thus correspond to units located at the periphery of the lignin macromolecule (Lapierre et al., 1988). Therefore, a localized attack on this periphery might produce the decrease of guaiacyl residues detected by thioacidolysis.

Grass lignocelluloses are more condensed than lignin recovered from coniferous or deciduous wood (Lapierre et al., 1986b). The relatively constant yield of recovered H and S residues suggests that the solubilisation process apparently does not involve major modifications in the degree of condensation of this material. However, the increase of the S/G ratio suggests that a structural change has occurred.

Although thioacidolysis is not aimed at the optimal recovery of ferulic units, which are known to be characteristic components of grass cell walls (Higuchi, 1985), the method revealed that APPL was enriched in ferulic units, compared to the whole cell wall sample. This interesting finding suggests that lignin fragments in APPL are closely associated, or even linked to hemicellulosic components which are ester-linked to ferulic acid (Scalbert et al., 1985). This is in good agreement with the carbohydrate analyses of APPL, which show the "hemicellulosic" nature of the carbohydrate fraction in APPL. Such an association might be at the origin both of the high solubility of APPL in neutral or alkaline medium and of its characteristic precipitation at low pH. Also, independent experiments reported previously, determined that *S. viridosporus* does not degrade ferulic acid (Rüttimann et al., 1987).

Quantitative analysis of wheat lignocellulose and APPL indicated that the latter contain about 20% of carbohydrates, which is in agreement with results obtained by other researchers (Crawford et al., 1983; McCarthy et al., 1986). The solubilisation process involves a general decrease in total sugars which can be ascribed primarily to a significantly lower glucose content in APPL. At the same time, an increase of galactose, arabinose and also mannose could be observed.

Recently, comparative analyses of barley lignocellulose and the soluble material released by different *Actinomycetes* have been performed (Zimmermann and Broda, 1989). Results obtained by these authors also indicate that the lignin-carbohydrate component of the solubilized material shows pronounced differences when compared to the initial substrate.

Bacteria isolated in this work exerted rather minor action on APPL. Spectrophotometric and chromatographic analyses of APPL after growth of the different strains did not show any modification of the substrate. Chemical analysis of the

same material established that some of these isolates grew at the expense of sugar moieties of the APPL, whereas others attacked its aromatic components. None of the strains introduced significant changes into both components of the substrate. When comparing the metabolic versatility of these strains with their specific effect on APPL, general although not absolute consistency of bacterial action on APPL and the other substrates can be found.

Our results, as well as those of other authors, strongly indicate that APPL are quite refractant to bacterial degradation. In addition, present available data on fungal action on APPL suggest that these microorganisms do not depolymerize extensively this substrate either (Pometto and Crawford, 1986; our unpublished results). As Broda and his collaborators have suggested, the soluble polymers derived from lignocellulose after attack of filamentous bacteria have a striking similarity to humic acids (McCarthy et al., 1986). It has generally been assumed that *Actinomycetes* are primarily involved in the process of natural humidification of plant residues (Goodfellow and Williams, 1983), although direct proof is lacking. Since neither bacteria nor ligninolytic fungi seem to degrade APPL efficiently, slow small scale modifications might finally turn them to part of humic material. The integrated action of several non-filamentous bacteria on APPL might carry out that conversion. Clearly, this exciting possibility deserves further study.

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