Is the solubilized product from the degradation of lignocellulose by actinomycetes a precursor of humic substances?

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Three actinomycetes (*Streptomyces* sp. EC22, *Streptomyces viridosporus* T7A and *Thermomonospora fusca* BD25) were assessed for their ability to degrade ball-milled wheat straw. All gave maximum levels of solubilized lignocellulose products (APPL) at the beginning of the stationary phase of growth (72–96 h). Low-molecular-mass aromatic compounds extracted from the APPL were analysed by reverse-phase and gas chromatography. Although the number of chromatographic peaks detected made identification of the products difficult, *p*-coumaric acid (4-hydroxycinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), gallic acid methyl ester (methyl-3,4,5-trihydroxybenzoate) and 4-methoxyphenol were recognized. The infrared spectra of the three strains were similar to the spectra of humic acids, with all APPL extracts showing carbonyl, amino, carboxyl, aliphatic and aromatic group vibrations. Also detected were peptide linkages of proteins. The results suggest a role for actinomycetes in the formation of humic substances in soils and composts.

Keywords: actinomycetes, acid-precipitable polymeric lignin, humic substances, lignocellulose degradation

INTRODUCTION

Lignocellulose, the main component of wheat straw, is composed of three polymers; lignin (10–30%, dry wt), cellulose (25–45%, dry wt) and hemicellulose (24–50%, dry wt) (Betts *et al.*, 1992). Cellulose is the most abundant natural compound in nature. It is a homopolymer of glucose linked by β -1,4-glycosidic bonds. Unlike cellulose, hemicellulose is a non-linear heteropolymer composed mainly of xylose but also containing other carbohydrates such as galactose, mannose, arabinose, glucuronic acid, rhamnose and fucose linked together predominantly by β -1,4-glycosidic bonds. These two polymers are physically encrusted with lignin, to which they are covalently linked through lignin–saccharide bonds that provide mechanical strength and rigidity to plant tissues. In plant cell walls, monophenolic compounds such as *p*-coumaric and ferulic acids are known to link arabinoxylans to lignin via ester bonds (Shibuya, 1984; Gubler *et al.*, 1985; Nishitani & Nevins, 1988). Lignin is synthesized in plants from coniferyl and coumaryl alcohols through radical coupling

mediated by plant peroxidases. This random chemical coupling results in a complex three-dimensional phenolic polymer with a variety of bonding arrangements giving aryl glycerol- β -aryl ether, non-cyclic benzyl aryl ether, biphenyl, diphenylether, phenylcoumaran, 1,2-diaryl-propane and other such structures (Crawford, 1981). As a result of this molecular complexity accentuated by covalent attachment to other plant polymers (e.g. cellulose and hemicellulose), lignin decomposition is the rate-limiting factor for the biological and chemical degradation of lignocellulose materials.

In natural terrestrial habitats (e.g. soils and composts), lignin is degraded by a complex microflora which includes both eukaryotic and prokaryotic organisms, such as white-rot fungi and actinomycetes, respectively. Most studies on the processes involved in lignin degradation have used the white-rot fungus *Phanerochaete chrysosporium*, because of the high ligninolytic activities which it exhibits. Mineralization of lignin by white-rot fungi is an established enzymic process which occurs during the secondary phase of growth under conditions of nitrogen or carbon starvation and is mediated by a number of different enzymes, of which lignin peroxidases and Mn²⁺-

Abbreviation: APPL, solubilized lignocellulose products.

dependent peroxidases are the most important (for a review, see Kirk & Farrel, 1987).

Actinomycetes are another group of organisms that can degrade lignin materials, although little is known about their lignocellulose-degrading mechanisms (Vicuña, 1988). Actinomycetes are Gram-positive bacteria which can be found on almost every natural substrate including soils and composts, fresh water basins, foodstuffs and the atmosphere (Waksman, 1967). Their hyphal growth is well suited for the colonization of plant biomass and they secrete a range of enzymes active against lignocellulose (McCarthy & Ball, 1991). Research on the biodegradation of lignin by actinomycetes has focused on the screening and detection of good lignocellulose degraders (Crawford, 1978; Crawford *et al.*, 1983; McCarthy & Broda, 1984; Ball & McCarthy, 1988; Ball *et al.*, 1989; Trigo & Ball, 1994). Even so, bacterial rates of lignin degradation compare poorly with the white-rot fungus *P. chrysosporium*. By following $^{14}\text{CO}_2$ evolution from the degradation of [^{14}C]lignin-labelled lignocellulose, McCarthy and co-workers could detect, in a number of actinomycetes, less than 10% lignin mineralization to CO_2 compared to 50% for *P. chrysosporium* (McCarthy & Broda, 1984; McCarthy *et al.*, 1984). This difference in lignin degradation rates between fungi and bacteria is probably due to different strategies of lignin degradation developed by these two groups of micro-organisms. Although lignin degradation by both bacteria and fungi is an enzymic process, bacterial degradation results in solubilization rather than depolymerization and mineralization of lignocellulose (McCarthy, 1987). The primary product from the degradation of straw by actinomycetes is a soluble high-molecular-mass, lignocarbohydrate complex (APPL) (Crawford *et al.*, 1983; Borgmeyer & Crawford, 1985; McCarthy *et al.*, 1986; Pometto & Crawford, 1986).

Crawford *et al.* (1983) first reported APPL production in culture supernatants of *Streptomyces viridosporus* T7A. While ^{14}C isotopic techniques confirmed the lignin origin of APPL (McCarthy *et al.*, 1986), other analyses showed chemical differences between APPL of inoculated cultures and uninoculated control samples (Crawford *et al.*, 1983). These differences included an increase in the number of phenolic hydroxyl and carboxyl groups in APPL, suggesting oxidative cleavage of *p*-hydroxy ether linkages and methoxyl groups in the lignin structure (Crawford *et al.*, 1983). Additional hydroxyl and carboxyl groups were also detected in APPL from *Streptomyces* sp. EC1 (Ball *et al.*, 1990). Analysis of APPL by solid-state ^{13}C -NMR spectroscopy (McCarthy *et al.*, 1986) showed an increase in carbonyl groups. Although McCarthy *et al.* (1986) found no solubilization of radiolabelled lignocellulose by *Thermomonospora mesophila* and *Streptomyces cyaneus* (suggesting that APPL was an end-product), Crawford and co-workers showed that *S. viridosporus* T7A, *Streptomyces badius* and the eukaryotic lignin-degrader *P. chrysosporium* could significantly degrade APPL produced by *S. viridosporus* T7A. Among the products formed by these organisms were *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), *p*-hydroxy-

benzoic acid, *p*-hydroxybenzaldehyde, protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid) and vanillin (4-hydroxy-3-methoxybenzaldehyde) (Pometto & Crawford, 1986). In addition, syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), vanillin and vanillic acid were detected in culture supernatants of *Streptomyces* sp. EC1 (Godden *et al.*, 1992). These data suggest that APPL is not simply an end-product but can be further modified. Furthermore, HPLC analysis of extracts from APPL in culture supernatants obtained at different times from *Streptomyces* sp. EC1 growing on wheat straw showed that modifications of APPL do occur during growth of the organism (Ball *et al.*, 1990).

The work described above is not conclusive regarding the fate of APPL in culture supernatants. These studies were carried out *in vitro*. In natural habitats, production of APPL by actinomycetes may be part of a more complex process, with other bacteria and fungi using APPL as substrates. In natural environments, APPL may serve as an intermediate in the formation of humic substances in soils and composts (Donnelly & Crawford, 1988; Senesi *et al.*, 1987; Fustec *et al.*, 1989). Humic substances are not defined in specific chemical terms, but as the fraction of organic matter that is not soluble in water at $\text{pH} < 2$ but soluble at neutral and alkaline pH (Amalfitano *et al.*, 1992). In natural terrestrial environments, several enzymes, including phenoloxidases and peroxidases, have been found associated with humic substances through covalent and hydrogen bonds (Martin & Haider, 1980; Sufita & Bollag, 1980; Sjöblad & Bollag, 1981). Similarly, Ball *et al.* (1990) detected veratryl alcohol and peroxidase activities in the APPL fraction extracted from supernatants of *Streptomyces* sp. EC1. The association of proteins with lignocellulose degraded products results in protection of the tertiary structure of proteins, which increases resistance to long term denaturation. (Skujins & McLaren, 1969; Pettit *et al.*, 1977). This long term action of native enzymes may meet the requirements for lignin degradation.

In this work we describe the characterization of APPL during growth on wheat straw of three previously used (Trigo & Ball, 1994) lignocellulolytic actinomycetes, and examine the relationship between APPL and humic substances.

METHODS

Organisms and growth conditions. Three strains of selected mesophilic and thermophilic actinomycetes were used: *Streptomyces* sp. EC22 (thermophilic), *Streptomyces viridosporus* T7A (mesophilic) and *Thermomonospora fusca* BD25 (thermophilic). They were obtained from W. Amner, University of Liverpool, D. L. Crawford, University of Idaho and A. J. McCarthy, University of Liverpool, respectively, and were maintained as suspensions of spores in 20% (v/v) glycerol at -20°C . Distilled water suspensions of sporulating growth on L-agar plates (Hopwood *et al.*, 1985) were used to inoculate shake flasks containing basal salts medium and 0.6% (w/v) yeast extract (McCarthy & Broda, 1984) supplemented with 0.2% (w/v)

ball-milled wheat straw. After inoculation, cultures were incubated at 37 or 50 °C with shaking at 200 r.p.m.

Measurement of acid precipitated polymer of lignin (APPL). Lignocarbhydrate solubilizing activity was determined by acidifying culture supernatants to pH 1–3 with HCl. The amount of APPL present was measured by recording the increase in absorbance at 600 nm over a 5 min period following acidification (Ball *et al.*, 1990; Trigo & Ball, 1994).

Extraction and analysis of APPL. Supernatants (100 ml) were acidified to pH 1–3 with HCl. After a 30–60 min interval, the APPL was extracted twice with ethyl acetate (40 ml) and once with ethyl ether (40 ml). The organic extracts were evaporated overnight (Pometto & Crawford, 1990).

(i) **Reverse-phase chromatography.** Low-molecular-mass organic-soluble compounds were extracted from APPL by leaching the sample with 500 µl 10% (v/v) acetonitrile in water. Following clarification by centrifugation, these solutions were chromatographed in a Beckman HPLC system composed of a programmable solvent module (Module 126) and a scanning detector (Module 167) equipped with a reverse-phase column (25 cm × 4.6 mm i.d.) of Spherisorb ODS2 (Anachem) and a 20 µl injection loop. The mobile phase was a gradient of acetonitrile in water adjusted to pH 1–3 with H₂SO₄. The acetonitrile concentration in the mobile phase started at 10% (v/v), increased to 50% (v/v) over 2 min and then to 100% (v/v) acetonitrile over the following 10 min. The flow-rate was 0.8 ml min⁻¹. Peaks were scanned for their UV spectra, and their retention time was recorded. Their spectral properties were compared to the standard compounds gallic acid, protocatechuic acid, *p*-coumaric acid (Sigma), 4-methoxyphenol and 2,6-dimethoxyphenol (Aldrich).

(ii) **Gas-chromatography.** The following procedure was used to extract low-molecular-mass organic-soluble compounds from APPL fractions: samples were derivatized with 300 µl dioxane, 20 µl pyridine and 10 mg trimethylsilylacetamide (TMSA, Sigma) for 2 h at 37 °C. After centrifugation to clarify the reaction mixture, samples (1 µl) were injected in a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector and an OV-1 capillary column (30 m × 0.32 mm). The oven temperature was maintained at 120 °C for 5 min, followed by a 10 °C min⁻¹ gradient to 260 and 280 °C. The carrier gas was helium.

Aromatic compounds extracted from APPL fractions were identified by comparing their retention times with 12 standard compounds (*p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, gallic acid methyl ester, caffeic acid, syringic acid (Sigma), acetovanillone, 3,5-dimethoxybenzoic acid, 2,6-dimethoxyphenol, isovanillic acid (3-hydroxy-4-methoxybenzoic acid) and 4-methoxyphenol (Aldrich).

(iii) **IR spectroscopy.** Infrared spectra of the APPL fractions were recorded for the wavelength range 4000–600 cm⁻¹ with a Perkin-Elmer infrared spectrophotometer model 1576. Samples were prepared by mixing with KBr and compressing the mixture into a disc (Kendall, 1966).

RESULTS AND DISCUSSION

Analysis of extracted solubilized degradation product from the growth of actinomycetes on wheat straw

During growth up to stationary phase, *Streptomyces viridosporus* T7A, *Streptomyces* sp. EC22 and *Thermomonospora fusca* BD25 produced APPL on ball-milled wheat straw. Maximum levels of APPL were recorded at

the beginning of the stationary phase (72–96 h) for all the strains examined (data not shown). Although all strains were able to solubilize straw, *T. fusca* BD25 produced the highest levels of APPL (OD₆₀₀ = 0.11). To investigate the composition and the variability of the solubilized product, APPL from the three strains was harvested after 72–96 h and low-molecular-mass organic-soluble compounds were extracted. These compounds were separated by reverse-phase HPLC and gas-chromatography. The HPLC chromatographic profiles of APPL produced by the three actinomycetes are shown in Fig. 1. All three strains were capable of solubilizing lignocellulose material in excess of the control (uninoculated flask). However the chromatographic profiles of the organisms examined were distinct. Although the intensities of chromatographic peaks for APPL extracts from the three strains were different, most peaks eluted between 2.5 and 9.0 min. The differences in chromatographic profiles may support the view that lignin is degraded by different mechanisms in different species of actinomycetes (Borgmeyer & Crawford, 1985; Mason, 1988).

UV spectra of the major peaks obtained during chromatography of compounds extracted from the APPL of each actinomycete and the peaks' retention times, were compared with those of commercially available standards. Only the product with a retention time of 2.8 min was found in cultures of all three strains; this was not identified. Although most of the other products were unique to each actinomycete strain, *p*-coumaric acid (retention time 7.9 min) was identified in *T. fusca* BD25 and *S. viridosporus* T7A. Also, gallic acid (retention time 5.7 min) was identified in *Streptomyces* sp. EC22 (Table 1). Analysis of the solubilized products present in APPL was also performed by gas-chromatography. Chromatographic profiles of the extracts from APPLs of the three strains showed an increase in the number of peaks compared to the control (uninoculated flask) (Fig. 2). Although most of the peaks on chromatograms were not conclusively identified, some of them matched the retention times of the standards used. On the basis of retention times, 4-methoxyphenol (retention time 1.48 min) was present in extracts of APPL from all strains, as were protocatechuic acid (retention time 9.86 min), 2,4-dimethoxybenzoic acid (retention time 7.72 min) and gallic acid methyl ester (retention time 10.76 min). Table 1 summarizes the compounds detected. Several aromatic products of straw degradation, including *p*-coumaric acid, were previously detected in *Streptomyces* sp. EC1 (Godden *et al.*, 1992), *Streptomyces badius*, *Streptomyces setonii* and *S. viridosporus* T7A (Pometto & Crawford, 1986). Esterified *p*-coumaric acid comprises a high percentage of the total weight of lignocellulose materials and is associated with lignin through ester linkages to the terminal γ -carbon of the phenylpropanoid unit of lignin (Shimada, 1972). These results favour the idea suggested by Pometto & Crawford that APPL is an intermediate that is further degraded to polymeric aromatic units, rather than the view that APPL is an end-product of a process by which cellulolytic and hemicellulolytic enzymes gain access to plant polysaccharides (Crawford *et al.*, 1984).

Fig. 1. HPLC reverse-phase chromatograms of low-molecular-mass organic-soluble extracts from APPL recorded at 270 nm after 72–96 h of growth for *S. viridosporus* T7A, *Streptomyces* sp. EC22 and *T. fusca* BD25.

Table 1. Compounds identified by HPLC and gas-chromatographic analysis of solubilized fractions from selected actinomycete strains

(T) Denotes thermophile; + indicates product was present in the chromatographic profile; – indicates product was absent from the chromatographic profile.

Structural formula	Standard compound	<i>Streptomyces</i> sp. EC22 (T)	<i>S. viridosporus</i> T7A	<i>T. fusca</i> BD25 (T)
	<i>p</i> -Coumaric acid	–	+*	+*
	Protocatechuic acid	+	–	–
	Gallic acid	+*	–	–
	Gallic acid methyl ester	+	+	–
	2,4-Dimethoxy benzoic acid	+	+	–
	4-Methoxyphenol	+	+	+

*The product was identified by reverse-phase HPLC. All other products were identified by gas-chromatography.

Fig. 2. Gas-chromatography analysis of low-molecular-mass organic-soluble extracts from APPL for *S. viridosporus* T7A, *Streptomyces* sp. EC22 and *T. fusca* BD25. Peaks identified on the basis of their retention times (min) are indicated (1.48, 4-methoxyphenol; 7.72, 2,4-dimethoxybenzoic acid; 9.86, protocatechuic acid; 10.76, gallic acid methyl ester).

Qualitative changes in the solubilized product during growth and solubilization of straw by *T. fusca* BD25

To examine changes in APPL during growth of *T. fusca* BD25 on ball-milled wheat straw, samples of APPL were taken from cultures at regular intervals. The composition of low-molecular-mass organic-soluble compounds was determined by HPLC (Fig. 3). After 3 d growth, significant amounts of aromatic material were present in APPL extracts. After 6 d incubation, the composition of these extracts showed distinct changes. After 9 d the

composition of the APPL extracts stabilized and remained unchanged during the following 7 d incubation. Similar results were obtained with APPL from *S. viridosporus* and *S. badius* (Borgmeyer & Crawford, 1985). *Streptomyces* sp. EC1 also changed in chromatographic profile when sampled at intervals during growth (Ball *et al.*, 1990). The latter authors suggested that depolymerization-polymerization occurs implying that APPL could serve as an intermediate in the formation of humic substances in composts.

Infrared spectroscopy analysis

Infrared spectral analysis of APPL from actinomycete culture supernatants (Fig. 4) showed considerable similarity between the products from the three strains. The spectra of all preparations displayed a strong band at $3400\text{--}3200\text{ cm}^{-1}$, probably due to stretching vibrations of H-bonded OH and NH_2 groups (Stevenson & Goh, 1971). In the case of *T. fusca* BD25, this band is shifted towards a lower wave number, perhaps reflecting the increased presence of H-bonded OH and NH_2 groups in the humic-type APPL polymers (Senesi *et al.*, 1987). Common to these three spectra were weak bands at $1740\text{--}1710\text{ cm}^{-1}$ attributed to numerous C=O containing groups (Russel *et al.*, 1983). Aliphaticity is shown by bands at 2850 cm^{-1} (aliphatic C-H stretch) and 1430 cm^{-1} (aliphatic C-H deformation) and 1000 cm^{-1} (skeletal vibration of aliphatics) (Senesi *et al.*, 1987). A broad band of medium intensity at 1660 cm^{-1} and small bands at $1530\text{--}1510\text{ cm}^{-1}$, shown by all three spectra, can be attributed to the peptide linkage in proteins (Stevenson & Goh, 1971; Filip *et al.*, 1974; Russel *et al.*, 1983), supporting previous reports that proteins are bound to APPL (Ball *et al.*, 1990). Also, there is evidence of adsorption of active enzymes to humic substances (Claus & Philip, 1988). Generally, the spectra from all APPL preparations showed a high degree of similarity with spectra of humic substances (Boyd *et al.*, 1979; Russel *et al.*, 1983; Senesi *et al.*, 1987), consistent with suggestions that actinomycetes contribute to humification (Ball *et al.*, 1990; McCarthy *et al.*, 1986).

In conclusion, the three strains examined were able to grow and solubilize ball-milled wheat straw. Further, the solubilized product from all three strains contained aromatic monomers, such as *p*-coumaric acid, protocatechuic acid, gallic acid, gallic acid methyl ester, 2,4-dimethoxybenzoic acid and 4-methoxyphenol. Phenolic acids are considered to play a major role in humification in soils (Morita, 1965; Katase, 1981). In fact, humic substances are predominantly formed by humic acid polymers which are synthesized from phenolic acids, amino acids and other large molecules through enzymic and oxidative reactions (Felbeck, 1971; Haider *et al.*, 1975; Martin & Haider 1980). Oxidative polymerization is thought to be mediated by phenoloxidases and peroxidases through the formation of phenoxy-radicals which are converted to stable compounds by self- or cross-coupling to other molecules (Bollag *et al.*, 1980, 1981; Martin & Haider, 1980). Production of extracellular peroxidase is common

Fig. 3. HPLC reverse-phase chromatograms of solubilized low-molecular-mass organic-soluble extracts from APPL from culture supernatants of *T. fusca* BD25 grown on

Fig. 4. Infrared spectral analysis of APPL from *T. fusca* BD25 (a), *Streptomyces* sp. EC22 (b) and *S. viridosporus* T7A (c). Culture samples were prepared in KBr as compressed tablets.

amongst actinomycetes (Trigo & Ball, 1994), suggesting that this enzyme may be involved not only in degrading lignin (Ramachandra *et al.*, 1988, Trigo & Ball, 1994), but also in polymerizing the resulting phenolic acids, proteins and sugars to yield a humic acid type polymer, APPL. This suggestion is also supported by the depolymerization-polymerization of lignin reported here for *T. fusca* BD25 and observed in several actinomycete strains (Borgmeyer & Crawford, 1985; Ball *et al.*, 1990). Finally, infrared spectroscopy confirmed the similarity of APPL to humic acids. These results suggest that the lower levels of lignin degradation by actinomycetes compared to fungi may allow this group of organisms to play a significant role in humification processes in natural soils and composts.

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