Metabolism of low molecular weight lignin-related compounds by *Streptomyces viridosporus* T7A

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The ability of the ligninolytic actinomycete Streptomyces viridosporus T7A *to degrade selected lignin model compounds, both in the presence and in the absence of lignocellulose, was examined. Compounds studied included benzyl alcohols and aldehydes, plus dimers possessing intermonomeric linkages, which are characteristic of the lignin macromolecule. Oxidation of veratryl alcohol to the corresponding acid was significant only under ligninolytic growth conditions,* i.e., *in medium containing lignocellulose, while other benzyl alcohols and aldehydes were readily oxidized in its absence.* S. viridosporus T7A *reduces carbonylic groups of 1,2-diarylethane, but not of 1,2-diarylpropane structures, under both ligninolytic and non-ligninolytic culture conditions. Cleavage of 1,2-diarylpropane (β-1), arylglycerol-β-arylether (β-0-4) and biphenyl structures by this strain could not be detected under either metabolic conditions.*

Keywords: *Streptomyces*; lignin related-compounds; lignocellulose

Introduction

Strains belonging to the genus *Streptomyces* are known to attack grass lignocellulose, generating a soluble lignin-rich product with molecular weight >20000.[1,2] This degradation product becomes insoluble when the pH of the medium is lowered with acid, and hence its name "APPL," for acid precipitable polymeric lignin.[1]

The enzymatic activities that are responsible for the release of APPL to the aqueous medium are unknown. Due to the close association of the aromatic polymer with carbohydrates in grass lignocelluloses, cellulases could play a role in this process. Indeed, the participation of these enzymes in APPL production from corn lignocellulose has recently been confirmed.[3] On the other hand, it is conceivable that lignin solubilization would also require direct rupture of intermonomeric linkages in lignin itself, such as *β-O*-4, *β*-1, diarylether, biphenyl, etc. So far, information concerning enzymes from *Streptomyces* catalyzing this type of reaction is very scant. There is a report of an enzyme from *Streptomyces viridosporus* T7A that is able to cleave the *β-O*-4 linkage of a dimeric lignin model substrate.[4] However, its role in lignocellulose breakdown has not been assessed.

Although degradation of lignin model compounds by a microorganism may not reflect true ligninolytic capacity, this approach has proved successful in studies with filamentous fungi. Therefore, in an effort to look for the presence of ligninolytic enzymes in cultures of *S. viridosporus* T7A, we examined the bioconversion of lignin-related compounds by whole cells of this strain under two culture conditions, namely ligninolytic *versus* non-ligninolytic. The former, consisting of medium containing wheat lignocellulose, allowed us to test for enzyme induction, if any, and also to follow the fate of the substrate while APPLs were being produced.

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Materials and methods

Chemicals

Anisic, caffeic, cinnamic, *p*-coumaric, ferulic and *p*-hydroxybenzoic acids were from Sigma Chemical Co., USA. Veratryl alcohol (compound I), anisyl alcohol (II), *o*-, and *m*- and *p*-anisaldehyde, catechol, guaiacol, veratraldehyde, gallic acid, benzoin (α-hydroxy-α-phenylacetophenone, compound IV), deoxybenzoin (2-phenylacetophenone, V), anisoin (4,4′-dimethoxybenzoin, VI), desoxyanisoin (4′-methoxy-2-(4-methoxyphenyl) acetophenone, VII), 4,4′-dimethoxybenzil (X), 4,4′-biphenol (4,4′-dihydroxybiphenyl, XXII), 3,3′-dimethoxybiphenyl (XXIII), 2,2-dimethoxy-2-phenylacetophenone (IX) and α-phenylcinnamic acid were from Aldrich Chemical Co., USA. Dihydrobenzoin (XI), deoxyhydroxybenzoin (XII), dihydroanisoin (XIII) and desoxyhydroxyanisoin (XIV) were prepared by $NaBH_4$ reduction at room temperature of compounds IV, V, VI and VII, respectively, in tetrahydrofuran containing 10% ethanol. 1,2-Bis (4-methoxyphenyl)-3-hydroxypropanone (VIII) and 1,2-bis (4-methoxyphenyl)-propane-1, 3-diol (XV) were prepared following the procedure

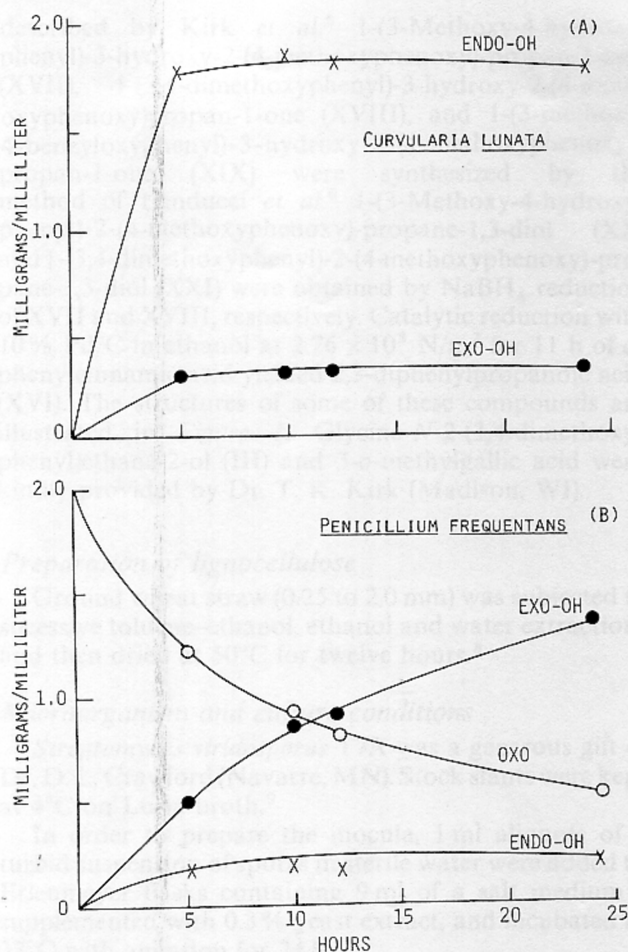

Figure 2 Kinetics of reduction of (+)-2-oxo-1,4-cineole by *C. lunata* (*A*) and *P. frequentans* (*B*) ○, 2-oxo-1,4-cineoles; ●, 2-exo-hydroxy-1,4-cineoles; and X, 2-endo-hydroxy-1,4-cineoles

when *8* is used as substrate with *P. frequentans*, it remains unchanged. Another possible explanation for this observation, that the endo-alcohol is further transformed to unidentified metabolites, is unreasonable based upon the mass balance of nearly 100% obtained at each assay time in this experiment. The results suggests the existence of multiple reductases in *P. frequentans* possibly formed or favored at different times during the course of this experiment.

Work with enantiomeric 2-oxo-1,4-cineoles demonstrated the selectivities of microbial reductases in achieving ketone reductions. Cultures such as *P. chrysogenum* and *Stemphylium consortiale* preferentially reduced one of the ketone isomers. Thus, it would be possible to use such microbial catalysts to accomplish differential enrichment of ketone enantiomers. Microbiological reductions catalyzed by growing cells may be subject to a wide variety of influences including age of culture, the physiological state of the microorganism during nutrient depletion and the presence of microbial enzymes capable of catalyzing competing reactions.

Acknowledgement

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described by Kirk *et al.*[5] 1-(3-Methoxy-4-hydroxyphenyl)-3-hydroxy-2-(4-methoxyphenoxy)-propan-1-one (XVII), 1-(3,4-dimethoxyphenyl)-3-hydroxy-2-(4-methoxyphenoxy)propan-1-one (XVIII), and 1-(3-methoxy-4-benzyloxyphenyl)-3-hydroxy-2-(4-methoxyphenoxy)-propan-1-one (XIX) were synthesized by the method of Landucci *et al.*[6] 1-(3-Methoxy-4-hydroxyphenyl)-2-(4-methoxyphenoxy)-propane-1,3-diol (XX) and 1-(3,4-dimethoxyphenyl)-2-(4-methoxyphenoxy)-propane-1,3-diol (XXI) were obtained by $NaBH_4$ reduction of XVII and XVIII, respectively. Catalytic reduction with 10% Pd/C in ethanol at 2.76×10^5 N/m^2 for 11 h of α-phenylcinnamic acid yielded 2,3-diphenylpropanoic acid (XVI). The structures of some of these compounds are illustrated in *Figure 1*. Glycine-*N*-2-(3,4-dimethoxyphenyl)ethane-2-ol (III) and 3-*o*-methylgallic acid were kindly provided by Dr. T. K. Kirk (Madison, WI).

Preparation of lignocellulose

Ground wheat straw (0.25 to 2.0 mm) was subjected to sucessive toluene–ethanol, ethanol and water extractions and then dried at 50°C for twelve hours.[8]

Microorganism and culture conditions

Streptomyces viridosporus T7A was a generous gift of Dr. D. L. Crawford (Navarre, MN). Stock slants were kept at 4°C on Luria broth.[9]

In order to prepare the inocula, 1 ml aliquots of a turbid suspension of spores in sterile water were added to Erlenmeyer flasks containing 9 ml of a salt medium[10] supplemented with 0.3% yeast extract, and incubated at 37°C with agitation for 24 h.

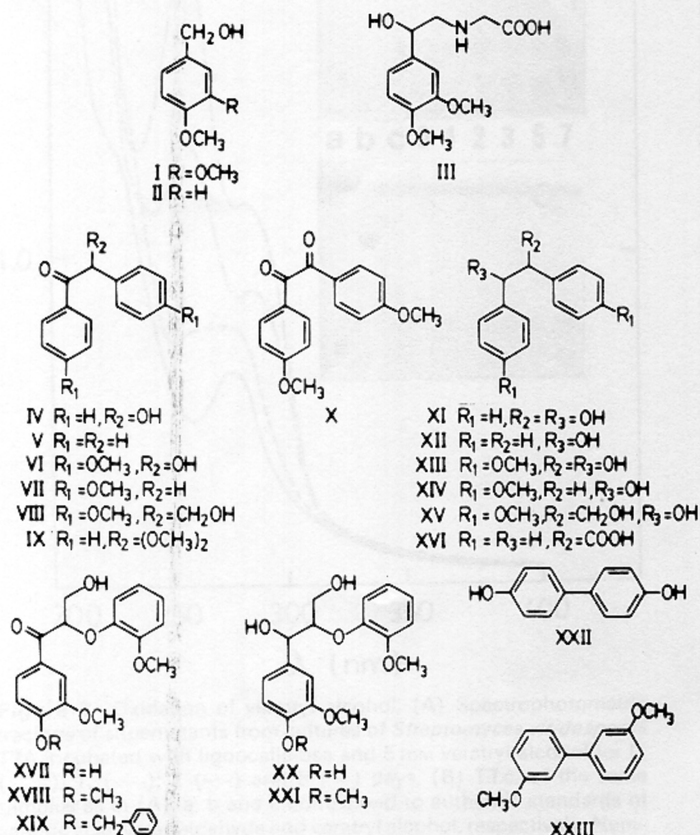

Figure 1 Molecular formulae of the lignin-related compounds used

Cultures with the lignin-related compounds were carried out under two different conditions: (a) 0.1 ml of the cell suspension was inoculated directly on 10 ml of the same minimal salt medium described containing 0.3% yeast extract and either 1.0–5.0 mM of single-ring aromatic substrate or 0.1–0.5 mg/ml of dimeric model compound, and shaken at 37°C, or (b) 0.1 ml of the inoculum was transferred onto 100 mg sterile wheat straw lignocellulose and grown on this solid substrate at 37°C for 96 h. At this time, 10 ml of the salt medium indicated in (a) containing the aromatic compound to be assayed were added and the flasks were further incubated with agitation at 37°C. Under these conditions solubilization of some of the dimeric substrates, mainly IX, X and XXIII was not complete.

One ml aliquots were taken for analytical purposes from either type of culture at the time intervals indicated in the text.

Analytical procedures

One ml samples were withdrawn from the cultures and centrifuged at 5000 rev min^{-1} for 5 min. The supernatants were acidified to pH 1.0–2.0 with 12N HCl, extractied twice with one volume of diethyl ether and the ether extracts evaporated to dryness.

Spectrophotometric analysis. Dried samples were resuspended in distilled water and absorbtion spectra between 200 and 400 nm were obtained in a Shimadzu u.v.-visible recording Spectrophotometer attached to a Shimadzu Graphic Printer PR-1 (Shimadzu Corp., Kyoto, Japan).

Thin layer chromatography (t.l.c.). Ether extracts from above were resuspended in 20 μl of diethyl ether and spotted on silica plates (Silica Gel 60F-254, Merck, Darmstadt). Occasionally, to prevent volatilization of some compounds during the evaporation of the solvent used for extraction, 0.1 ml of the acidified culture supernatants was extracted with one volume of diethyl ether and 1.0–20 μl of the organic phase were spotted directly on the plates. This was the case with veratryl alcohol, anisyl alcohol, guaiacol and *p*-anisaldehyde. Gallic and 3-*O*-methyl gallic acids were separated with the solvent system benzene: acetic acid (2:1). Cinnamic and ferulic acids were resolved using chloroform:acetone (20:7) and compound III was run in chloroform:acetone (40:1). The developing solvent for the *β-O-*4 compounds was chloroform:isopropanol:ammonia (12:1:1). All other compounds were run in chloroform:acetone (9:1). Aromatic compounds were visualized with short-wave u.v. light and photographed using 55 positive/negative, 50 ASA/18 Din Polaroid film.

High performance liquid chromatography (HPLC). Dried samples were solubilized with distilled water and injected in a Gilson (Middleton, WI, USA) System 41 Gradient Analytical HPLC apparatus, with an HM/HPLC detector employing a Hewlett-Packard (Palo Alto, CA, USA) 3380A integrator and Vydac (Hesperia, CA, USA) reversed-phase Ultrasphere ODS column. Samples were resolved with a water–methanol gradient, in which the concentration of the latter varied in time as follows: 0–5 min, 20%; 5–14 min, gradual increase from 20 to 40%; 14–32 min, 40%; 32–33 min, decrease to 20%; 33–45 min, 20%.

APPL quantification

Production of APPL was determined by measuring absorbance at 600 nm of acidified supernatants from aliquots taken from cultures containing lignocellulose, as originally described.[1]

Results

Oxidation of veratryl alcohol

When veratryl alcohol (I) was added to cultures of *S. viridosporus* T7A in salt medium supplemented with 0.3% of yeast extract, a minor conversion to veratric acid could be detected. In contrast, when the growth medium contained lignocellulose, oxidation of the alcohol to the acid was quantitative (*Figure 2*). These results suggest that some enzymes are induced when the bacterium is proliferating in the presence of lignocellulose. Veratryl alcohol was the only substrate tested that underwent different metabolic fates under the two culture conditions. Veratric acid accumulated in the medium and was not further metabolized, as reported previously.[11]

Metabolism of other single-ring aromatic compounds

Compound II (*p*-anisyl alcohol) was converted to the corresponding acid, which accumulated in the medium

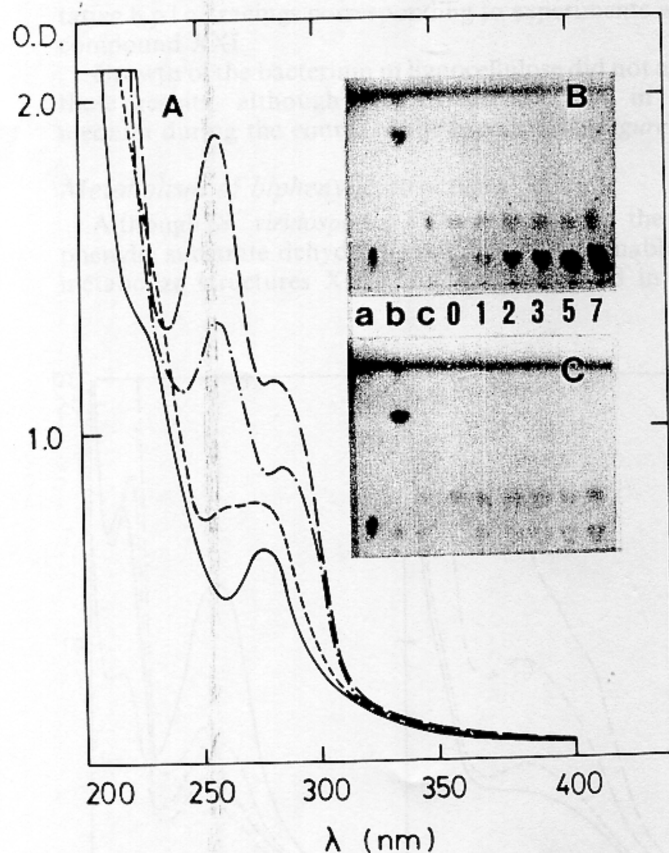

Figure 2 Oxidation of veratryl alcohol. (A) Spectrophotometric tracings of supernatants from cultures of *Streptomyces viridosporus* T7A incubated with lignocellulose and 5 mM veratryl alcohol for 0, (——); 1, (----); 2 (–·–) and 5 (-–-) days. (B) T.l.c. of the same samples as in (A): a, b and c correspond to authentic standards of veratric acid, veratraldehyde and veratryl alcohol, respectively. Numbers indicate incubation time in days. (C) T.l.c. of samples taken from cultures of *S. viridosporus* T7A with veratryl alcohol incubated in the absence of lignocellulose: a, b, c and numbers are as in (B)

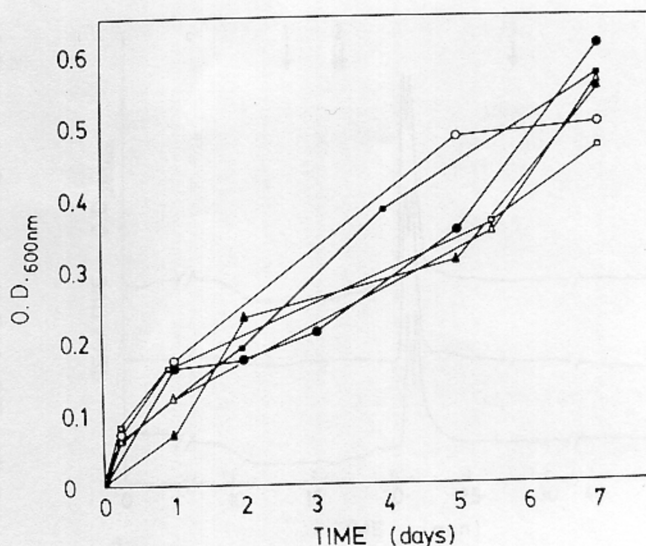

Figure 3 APPL production from wheat lignocellulose by *Streptomyces viridosporus* T7A in the presence of different aromatic compounds: (○), XVII; (■), coumaric acid; (□), X; (△), XXII; (▲), catechol and (●), no aromatic substrate added

(data not shown). In this experiment, *p*-anisaldehyde was not detected, indicating that it was rapidly oxidized to *p*-anisic acid. When *p*-anisaldehyde was added as the starting substrate, conversion to *p*-anisic acid was also observed. We found that the microorganism discriminates between the isomers of anisaldehyde: *p*-anisaldehyde (5 mM) was readily converted to *p*-anisic acid, while *o*-anisaldehyde (5 mM) inhibited bacterial growth. However, at a lower concentration (1 mM), it was partially metabolized to *O*-anisic acid. In turn, *m*-anisaldehyde (1 mM) was also partially oxidized to *m*-anisic acid. The benzoic acids syringic, gallic and 3-*o*-methyl gallic as well as the phenylpropanoic acids cinnamic, coumaric and ferulic—all assayed at 5.0 mM—and guaiacol (1.0 mM) were not metabolized by *S. viridosporus* T7A.

To explore whether metabolism of these aromatics takes place while this bacterium is degrading lignocellulose, the compounds were added to cultures in the presence of purified sterile wheat lignocellulose as indicated in Methods. Incubation in this medium leads to lignin solubilization, as indicated by the accumulation of APPLs (*Figure 3*). However, compounds that had not been metabolized in the absence of lignocellulose also remained unmodified in this new medium.

Similar experiments were designed for compound III. Tien *et al.*[7] have described that ligninase from the fungus *Phanerochaete chrysosporium* cleaves this adduct, producing glycine, veratraldehyde and formaldehyde. Results showed that *S. viridosporus* T7A fails to modify this compound under either metabolic condition.

Metabolism of 1,2-diarylethane and 1,2-diarylpropane structures

Shimada *et al.*[13] used anisoin (VI) as a β-1 model substrate. They showed that *P. chrysosporium* cleaves the C–C intermonomeric linkage of anisoin only after its ligninolytic system has been induced. We found that in cultures of *S. viridosporus* T7A, reduction of the carbonyl group of anisoin took place. This was demonstrated by a shift from the spectrum of anisoin to that characteristic of

dihydroanisoin (XIII) *Figure 4A*) and confirmed by t.l.c. Compound X, possessing two carbonyl groups, was also reduced to dihydroanisoin, as shown by the same criteria. In this experiment an increase in u.v. absorbance was observed upon incubation (*Figure 4B*), which can be explained by a higher solubility of dihydroanisoin (XIII) with respect to that of dimethoxybencil (X). Dihydroanisoin was not further metabolized with longer incubation times.

Other compounds structurally related to anisoin that were readily transformed to their reduced forms by the bacterium were IV, V and VII. In contrast, compounds IX and the more lignin-like VIII were not reduced. The latter, as well as XVI, possess a γ-carbon that is absent in all the other β-1 models tested.

In no case was cleavage detected with any of the above substrates. Simultaneous degradation of lignocellulose by the actinomycete did not change this pattern.

Metabolism of model compounds with the arylglycerol-β-arylether linkage

The β-*O*-4 linkage is the most frequent one in the lignin polymer.[14] Of the compounds used in this work, XVII to XXI can be classified as β-*O*-4 models. They include both phenolic and non-phenolic dimers, and a "trimer" (XIX). When tested as substrates for *S. viridosporus* T7A, no modification could be observed by any of the analytical techniques used. As an example, *Figure 5* shows representative h.p.l.c. tracings corresponding to experiments with compound XXI.

Growth of the bacterium in lignocellulose did not alter these results, although APPLs accumulated in the medium during the course of the experiment (*Figure 3*).

Metabolism of biphenylic structures

Although *S. viridosporus* T7A can degrade the biphenylic substrate dehydrodivanillin,[15] it was unable to metabolize structures XXII and XXIII assayed in the present work. Again, when the incubation was performed in media with lignocellulose, APPLs were continuously being formed (*Figure 3*).

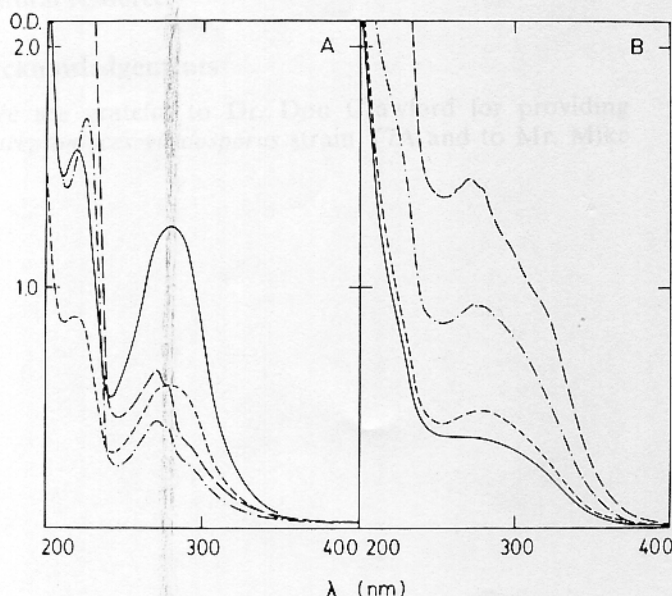

Figure 4 Reduction of 1,2-diarylethane structures. Spectrophotometric tracings of aliquots taken from cultures of *Streptomyces viridosporus* T7A grown in the presence of (A) anisoin (VI) and (B) dimethoxybenzil (X), at times 0, (——); 6, (---); 22, (-·-); and 94, (--) h of incubation

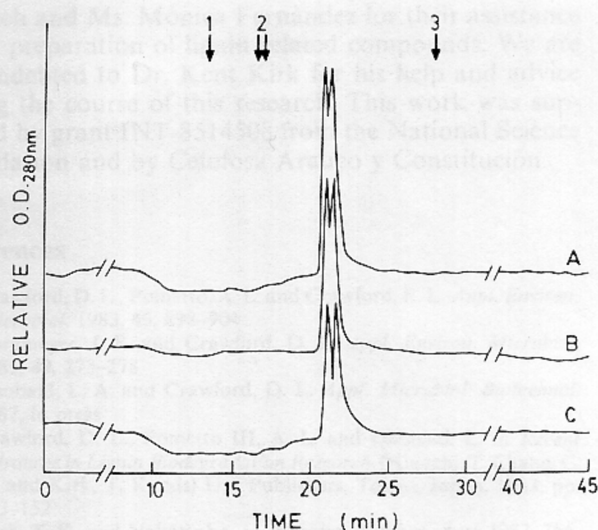

Figure 5 HPLC elution profiles of samples from cultures of *Streptomyces viridosporus* T7A grown in the presence of compound XXI. (A) and (B): aliquots withdrawn from cultures lacking lignocellulose, after 0 and 220 h of incubation; (C): aliquot withdrawn from a culture containing wheat lignocellulose, after 220 h of incubation. A double peak was observed due to the separation of the *erythro* and *threo* forms of compound XXI. The arrows indicate elution times of: guaiacol (1), *erythro* and *threo* forms of compound XX (2) and compound XVIII (3)

Discussion

Two important features of this report are the findings that *S. viridosporus* T7A is able to modify more single-ring aromatic substrates than those previously assayed, and that its action on dimeric lignin model compounds seems to be extremely limited, at least under the conditions employed.

That veratryl alcohol was the only substrate that was metabolized to a higher extent under ligninolytic conditions suggests that the enzymatic machinery involved in its oxidation is induced upon degradation of lignocellulose. Crawford *et al.*[11] have purified from *S. viridosporus* T7A a constitutive aldehyde oxidase that catalizes *in vitro* oxidation of veratraldehyde to the corresponding acid. This may indicate that the inducible enzyme in this system is the alcohol oxidase. Veratryl alcohol plays a key role in lignin biodegradation by white-rot fungi. This aromatic alcohol is released to the extracellular fluid in cultures of *P. chrysosporium*[16] and its synthesis marks the onset of secondary metabolism.[17] Other species of Basidiomycetes also produce this metabolite during ligninolysis (E. Agosin, personal communication). Moreover, Faison *et al.*[18] have shown that veratryl alcohol regulates the synthesis of ligninase. The significance of the finding that veratryl alcohol is oxidized during APPL production by *S. viridosporus* T7A remains to be established.

Cleavage of β-1 structures by this strain has not been reported and when tested in this work, it was not detected. With respect to cleavage of a dimeric substrate possessing the β-*O*-4 linkage, Crawford *et al.*[4] found that compound XXI was cleaved *in vivo*, leading to guaiacol accumulation in the medium. The enzymatic activity catalysing this reaction was induced by lignocellulose, and after partial

purification it remained associated with the insoluble fraction of the cells. In our hands, *S. viridosporus* T7A did not do so, even after growth on dampened wheat lignocellulose. This different behavior might be related to the type of lignocellulose used to induce the cleaving activity. An alternative explanation is that the enzyme may be coded by a plasmid that our strain, originally donated by Dr. D. Crawford, lost after several transfers. We have looked for extrachromosomal DNA in *S. viridosporus* T7A following the technique of Dobritsa[19] and have found none. Other modifications of the β-*O*-4 compounds XVII–XXI, such as demethoxylation or Cα-oxidation/reduction, were not detected either. An etherase activity able to remove methyl groups from the 4-position of veratryl moieties of XVIII and XXI, or the benzyl group in XIX, would have been expected, since APPLs are enriched in phenolic hydroxyl groups with respect to the lignin they are produced from.[1] This activity has already been shown to exist in *S. setonii* which demethylates vanillic acid, guaiacol and veratrol.[20]

It might be argued that at least in some cases, we may have overlooked metabolic intermediates due to a short period of transient accumulation. This would be valid for the β-*O*-4 compounds, as well as for the biphenyl and some of the β-1. We have ruled out this possibility since no consumption of the compound tested was observed.

At this point, it is not clear whether *S. viridosporus* T7A possesses an enzymatic system that is able to cleave intermonomeric bonds in lignin. Perhaps an assay with synthetic lignin[21] would provide a definite answer. Regardless of the outcome of this experiment, there is ample proof that Actinomycetes can efficiently attack grass lignocellulose. So far, participation of cellulases in lignin solubilization by *Streptomyces* has been demonstrated,[3,22] although their activities do not correlate with APPL production by *Thermomonospora mesophila*.[23] Further work is necessary to reach a better understanding of the action of Actinomycetes on lignin. This knowledge would be relevant not only for academic purposes but also to bring about an improved utilization of this abundant natural resource.

Acknowledgements

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