Appl Microbiol Biotechnol (1986) 24:347—352

Applied and Microbiology Biotechnology

Lignin solubilisation by *Thermomonospora mesophila*

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Summary. *Thermomonospora mesophila* degraded [^{14}C]lignin-labelled wheat lignocellulose to yield high molecular weight water-soluble products and a small amount of $^{14}CO_2$. Solubilisation of [^{14}C]lignin was found to be extracellular and inducible by growth on lignocellulose (straw) and hemicellulose (xylan), but was not correlated with xylanase or cellulase production.

The acid-precipitable product of straw degradation by *T. mesophila* was found to be a complex of lignin, pentose-rich carbohydrate and protein with some similarity to humic acids. Solid-state ^{13}C-NMR spectra of the dried product were generally similar to those of chemically extracted milled straw lignin but showed an increased content of carbonyl groups.

The relationship between degradation and solubilisation of lignin is discussed and a role suggested for actinomycetes in humification and the exploitation of lignocellulose bioconversion.

Introduction

Actinomycetes are much less efficient at converting lignin to CO_2 than white-rot fungi, such as *Phanerochaete chrysosporium*. However, it has been shown that certain actinomycete strains are able to readily solubilise the lignin component of grass lignocellulose, and this may be an important activity in the process of humification. While exploitation of this property in the bioconversion of lignocellulose to useful products is attractive, the biochemical basis of actinomycete attack on lignin has yet to be established.

Initial studies using [^{14}C]lignin-labelled lignocellulose have identified a range of lignin-degrading actinomycetes (Crawford 1978; Haider et al. 1978; Phelan et al. 1979; McCarthy and Broda 1984). Degradation of lignin by a strain of *Streptomyces viridosporus* yields both high molecular weight and low molecular weight products (Crawford 1981; Crawford et al. 1983), and a pathway for lignin catabolism involving a number of reactions including side-chain oxidation, demethylation of aromatic rings, and cleavage of β-ether bonds has been proposed (Crawford and Crawford 1984). Actinomycete ligninolytic activity is clearly different from that catalysed by the lignin-ase of *P. chrysosporium* (Kersten et al. 1985; Harvey et al. 1985) and furthermore, there is evidence that even within the genus *Streptomyces*, different mechanisms of lignin degradation have evolved (Borgmeyer and Crawford 1985).

Nuclear magnetic resonance spectroscopy of ^{13}C in solid samples using cross polarisation and magic angle spinning (CP/MAS NMR) is a relatively new technique in its application to lignocellulose structural determinations. Semi-quantitative information on certain aspects of lignin structure can be produced (Taylor et al. 1983) and the technique has been used to study modifications to lignins (Schaefer et al. 1981).

Thermomonospora mesophila degrades lignin to CO_2 and water-soluble products during primary growth on [^{14}C]lignin-labelled wheat lignocellulose (McCarthy and Broda 1984). In this paper, we report on the composition of the major soluble product and use CP/MAS NMR spectra

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to indicate the probable mechanism of attack. We also demonstrate that lignin solubilisation is an inducible, extracellular activity in *T. mesophila*.

Materials and methods

Bacterial strains and culture conditions. Thermomonospora mesophila DSM43048, *Thermomonospora fusca* MT816 and *Saccharomonospora viridis* NCIB9602 were maintained as suspensions of spores and hyphae in 20% (v/v) glycerol at −80°C. The strains were cultured routinely on CYC agar (Cross and Attwell 1974) adjusted to pH 8.0 and aqueous suspensions of sporulating growth used as inocula. A basal medium (M6) containing 1.0 $g \cdot l^{-1}$ yeast extract (McCarthy and Broda 1984) was used for liquid cultures. *Thermomonospora mesophila* cultures and cell-free preparations were incubated at 37°C, and those of the other two strains at 50°C.

Preparation of culture lysates and supernatants. Thermomonospora mesophila was grown up in 100 ml flasks containing 20 ml of medium, incubated with shaking for 11 d. The medium was supplemented with 0.05% (w/v) of one of the following: oat spelt xylan (Sigma Ltd.); vibratory ball-milled straw (mean particle size 5 μm in diameter); Indulin AT (Westvaco Corp., N. Charleston, S. Carolina); D-glucose; ferulic acid. Cultures were sonicated for 60 s before addition of lysozyme (1 mg ml^{-1} final concentration) and incubation at 37°C for 3 h. Lysates were sonicated for a further 90 s and the crude extracts clarified by centrifugation before passage through a membrane filter (0.45 μm pore diameter).

Crude extracellular preparations of all three strains were produced from cultures on the same medium with or without 0.1% (w/v) D-glucose, xylan or ball-milled straw. After incubation for 4 d, cultures were shaken with glass balls (3.4—4.5 mm diameter) at 200 rpm to release any enzyme loosely bound to culture solids. Culture supernatants were harvested by centrifugation and 0.03% (w/v) sodium azide added prior to storage at 4°C for up to 7 d. The xylanase activity of culture supernatants was determined by reducing sugar analysis as described previously (McCarthy et al. 1985).

Assay for [^{14}C]lignin solubilisation. Thermomonospora mesophila was cultured on [^{14}C]lignin-labelled wheat lignocellulose for 14 d and $^{14}CO_2$ evolution and ^{14}C solubilisation determined as described previously (McCarthy and Broda 1984). The culture supernatant was concentrated by lyophilisation and resuspension in approximately 1 ml distilled water. The concentrate was adjusted to pH 1.5 with 12 M HCl and the insoluble precipitate removed by centrifugation and redissolved in 0.5 ml dimethylformamide + 0.5% (v/v) acetic acid. A 0.2 ml aliquot of the supernatant was freeze-dried and resuspended in 0.2 ml of the same. These ^{14}C-labelled acid-soluble and insoluble fractions were analysed by HPLC in a Zorbax PSM60s gel permeation chromatography column (McCarthy et al. 1984).

Thermomonospora mesophila culture lysates and culture supernatants of all three strains were incubated with [^{14}C]lignin-labelled wheat lignocellulose and solubilised radioactivity determined after six and two days incubation, respectively.

Analysis of acid-insoluble products of straw degradation by T. mesophila. Thermomonospora mesophila was grown in 250 ml flasks containing 75 ml basal medium supplemented with one of the following: ball-milled straw (5 $g \cdot l^{-1}$); Kek-milled straw (fibres 1—5 mm × 0.1—0.5 mm) (5 $g \cdot l^{-1}$); Indulin AT (1.5 $g \cdot l^{-1}$); oat spelt xylan (2 $g \cdot l^{-1}$); D-glucose (1.5 $g \cdot l^{-1}$). After 7 d incubation with shaking, culture supernatants were harvested by centrifugation and adjusted to pH 1.5 with 12 M HCl. The precipitated material was collected by centrifugation, freeze-dried and weighed. Extracellular protein in culture supernatants, before and after acidification, was measured with a Bio-Rad protein assay kit (Bradford 1976). The acid-insoluble material prepared from the *T. mesophila* culture on ball-milled straw was subjected to a number of analyses. Elemental compositions of carbon, hydrogen and nitrogen were determined by the Microanalysis Laboratory, Department of Chemistry, UMIST using standard methods. Carbohydrate content was estimated by the anthrone colorimetric method (Morris 1948) with measurement of absorbance at 540 nm calibrated with D-glucose. Pentose content was estimated by the orcinol method (Mejbaum 1939) with measurement of absorbance at 665 nm calibrated with D-xylose. Carboxymethylcellulose and oat spelt xylan were used to confirm that these methods were quantitative, and in the case of the latter, specific.

CP/MAS ^{13}C-NMR spectroscopy. Samples of ball-milled straw, the acid-insoluble product of straw degradation by *T. mesophila* and milled straw lignin, prepared as described by Paterson et al. (1984), were analysed. All samples were run on a modified JEOL 200 MHz spectrometer with a ^{13}C frequency of 50.1 MHz and 4 K data points at British Petroleum Research Centre, Sunbury-on-Thames, Berkshire, England. All spectra were broad-band proton decoupled with a radio-frequency field of noise bandwidth 1.5 kHz. The decoupling field was applied with a radio-frequency amplitude of 10 gauss at 0.5 μs after the end of the cross-polarisation pulse and was maintained for the duration of the ^{13}C-magnetisation.

Results and discussion

Solubilisation of [^{14}C]lignin by T. mesophila

In 14 days growth at 37°C, the *T. mesophila* culture converted 7.5% of the [^{14}C]lignin-labelled wheat lignocellulose to $^{14}CO_2$ and solubilised 43% of the radiolabelled material. These are similar to values reported previously for this organism (McCarthy and Broda 1984) where it was observed that the solubilised ^{14}C-labelled products were relatively resistant to further degradation to CO_2. Approximately 50% of the total product, as determined by liquid scintillation counting, was precipitated by acid and at least 70% of this precipitate was soluble in the gel permeation chromatography solvent (dimethylformamide containing 0.5% v/v acetic acid). The results of the chromatographic analysis of both acid-soluble and acid-insoluble fractions are presented in Fig. 1. Gel permeation chromatography of lignins by HPLC is complicated by the elution of derivatised lignin model compounds at different positions from those predicted by column calibration with polystyrene standards (Pellinen and Salkinoja-Salonen 1985). This is demonstrated by the elution

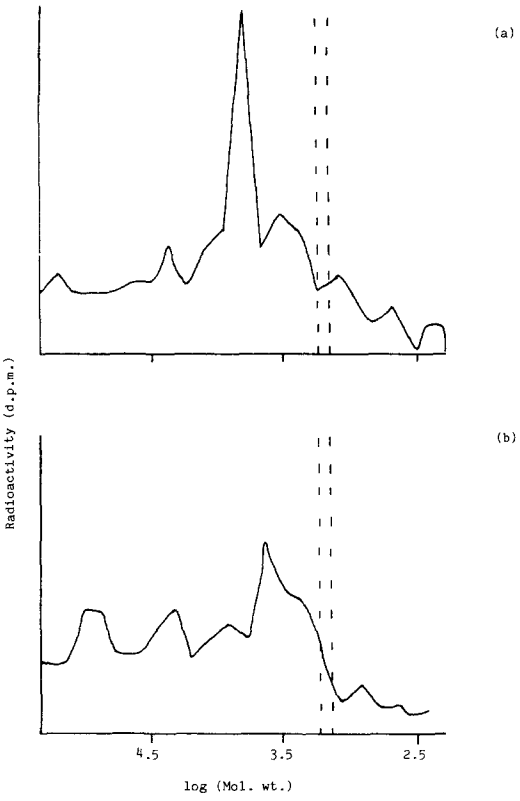

Fig. 1. Molecular weight range of the products of [^{14}C]lignin-labelled wheat lignocellulose degradation by *T. mesophila*. (**a**), acid-insoluble fraction; (**b**), acid-soluble fraction. The GPC column was calibrated with polystyrene molecular weight standards; the *dashed lines* represent, from left to right, the elution positions of α-conidendrin (dimer) and anisic acid (monomer) markers

Table 1. Solubilisation of ^{14}C-lignin by *T. mesophila* cell-free preparations

Growth substrate	^{14}C solubilised (% of total)
D-Glucose	8
Ferulic acid	4
Indulin AT	11
Ball-milled straw	22
Oat spelt xylan	21

Preparations were lysates of whole cultures on M6 broth + 0.05% (w/v) substrate, incubated with ^{14}C-lignin labelled wheat lignocellulose for 6 days at 37° C. The uninoculated control solubilised 6% of the total ^{14}C

positions of monomeric (p-anisic acid) and dimeric (α-conidendrin) model lignin compounds (Fig. 1), but it can nevertheless be concluded that most of the radiolabelled material solubilised by *T. mesophila* has a molecular weight >5000 daltons. This contrasts with results obtained in similar experiments with *P. chrysosporium,* where the products of lignin degradation were totally acid-soluble and eluted at a similar position to the monomeric and dimeric lignin models (McCarthy et al. 1984). The products of lignin degradation by *Streptomyces* spp. have also been reported as largely acid-insoluble and of high molecular weight (Crawford et al. 1983; Borgmeyer and Crawford 1985), suggesting that this type of product may be a general feature of actinomycete attack on lignocellulose.

Solubilisation of [^{14}C]lignin by cell-free preparations

The data presented in Table 1 show that lignin solubilisation can be obtained in the absence of whole cells. Furthermore, the enhancement of this activity by growth on lignocellulose or hemicellulose, but not by growth on either ferulic acid (3-methoxy, 4-hydroxy cinnamic acid), a lignin-related compound, or an industrial Kraft lignin (Indulin AT) suggests that it is inducible. The regulation of lignin-solubilising activity in *T. mesophila* thus appears to differ from that in *S. viridosporus* where it has been proposed that lignin itself acts as an inducer of an unidentified ligninolytic enzyme complex (Crawford and Crawford 1984). In both organisms, lignin solubilisation does not require H_2O_2 which is a fundamental requirement of the *P. chrysosporium* ligninase (Tien and Kirk 1983).

The observation that growth on xylan induces ligninolytic activity in *T. mesophila* suggests a role for xylanases in lignin solubilisation. In graminaceous cell walls, lignin is covalently bound to hemicellulose in the form of a lignocarbohydrate complex (Lai and Sarkanen 1971; Atushi et al. 1984). Consequently, xylanase attack on the carbohydrate moiety of this complex could be involved in lignocarbohydrate solubilisation. However, no correlation could be found between [^{14}C]lignin solubilisation and xylanase activity in *T. mesophila* culture supernatants, all of which contained relatively low amounts of xylanase activity (Table 2). Furthermore, thermophilic actinomycete culture supernatants which contained highly active xylanases gave [^{14}C]lignin solubilisation values <40% of those achieved by supernatants of *T. mesophila* cultures on xylan or straw (Table 2). Cellulase activity can also be discounted as contributing significantly to lignin solubilisation as *T. mesophila* supernatants contained 0—0.07 units ml^{-1} cellulase (carboxymethylcellulase) activity, and *Trichoderma reesei* cel-

Table 2. Solubilisation of ^{14}C-lignin by culture supernatants

Organism	Growth substrate	Xylanase activity (units ml^{-1})	^{14}C solubilised (% of total)
T. mesophila	D-Glucose	0.11	13
	Oat-spelt xylan	0.14	31
	Ball-milled straw	0.28	30
T. fusca	—	0.26	4
	Oat spelt xylan	3.40	7
S. viridis	Oat spelt xylan	1.89	11

Supernatants prepared from cultures on M6 broth plus 0.1% (w/v) substrate, incubated with ^{14}C-lignin labelled wheat lignocellulose for 48 h at 37° C *(T. mesophila)* or 50° C *(T. fusca* and *S. viridis)*. Uninoculated controls solubilized 1% and 3% of the total ^{14}C, respectively

lulase has been shown to solubilise only 10% approx. of the radioactivity in this substrate, attributed to the release of low molecular weight phenolics bound to carbohydrate in the graminaceous cell wall (McCarthy et al. 1984). The suggestion therefore is that growth of *T. mesophila* on xylan induces production of unidentified enzymes involved in lignin solubilisation. Whether induction is due to xylan degradation products or lignin-derived material often present in commercial xylan preparations, has yet to be determined.

Characterisation of the acid-precipitable product of straw degradation by T. mesophila

Studies on [^{14}C]lignin-labelled lignocellulose are useful for the identification of ligninolytic organisms and in preliminary characterisation of the physiology of lignin degradation. However, analysis of products solubilised from mature senescent lignocellulosic substrates such as straw is of more relevance to both natural degradative processes and biotechnological applications. *Thermomonospora mesophila* cultures on straw yielded significant quantities of solubilised acid-precipitable product corresponding to the polymer APPL (acid-precipitable polymeric lignin) which is an important product of lignocellulose degradation by streptomycetes (Borgmeyer and Crawford 1985). No acid-precipitable material was recovered from culture supernatants of *T. mesophila* grown on D-glucose, xylan or Kraft lignin (Indulin AT), confirming that the material originated from straw. A small amount (2 mg g^{-1} straw) was recovered from the uninoculated controls compared with the average yield of 72 mg/g^{-1} straw in *T. mesophila* cultures. Vibratory ball-milling disrupts the cell walls of the mature straw, providing the organism with ready access to the lignocellulose substrate. In experiments with ground (Kek-milled) straw, in which cell walls are more likely to remain intact, a yield of only 30 mg product per g straw was obtained.

The anthrone test for total carbohydrate gave an estimate of 20% carbohydrate in the acid-precipitable product. This carbohydrate appeared to comprise 80—90% pentose, as determined by the orcinol method, providing further evidence for the predominance of hemicellulose in the carbohydrate fraction of the grass lignocarbohydrate complex. The heterogeneous nature of the acid-precipitable material was further indicated by the elemental analysis: 51.4% carbon; 5.8% hydrogen; 4.2% nitrogen. The *T. mesophila* culture supernatants contained approximately 20 mg·protein per gram straw, which disappeared after acidification. In cultures grown in the absence of lignocellulose, extracellular protein was not precipitated by the acidification step. This suggests that acidification led to coprecipitation of protein and lignocarbohydrate to give a product which is similar in elemental analysis and acid-insolubility to humic acids (Hayes and Swift 1978). An association between degraded lignin and microbial protein has been reported previously in a study of lignin degradation by Gram-negative bacteria (Sørensen 1962). The ability of *T. mesophila* to produce material resembling humic acids, in pure culture on straw, supports the view that actinomycetes are intimately involved in the natural humification process.

Solid-state ^{13}C-NMR spectroscopy (CP/MAS NMR) is able to give valuable information on the structure of complex insoluble materials such as lignocellulose, but is limited in its ability to provide quantitative data. Spectra from ball-milled straw, acid precipitable actinomycete-solubilised product and milled straw lignin are presented in Fig. 2. In the ball-milled straw spectrum, carbohydrate signals predominate whereas in both the milled straw lignin and actinomycete product, the dominance of signals corresponding to lignin carbons is clearly evident. The intense signals from lignin C3 and C5 aromatic methoxyl groups suggest that *T. mesophila* has not extensively demethylated lignin during straw degradation, but this must be confirmed by specific determinations of the methoxyl content of extracted and degraded lignins. ^{13}C-NMR spectra of milled grass lignins have previously been shown to contain a signal at approximately 175 ppm, corresponding to the carbonyl groups of phenolic esters (Nimz et al. 1981). The peak at this chemical shift value can also be

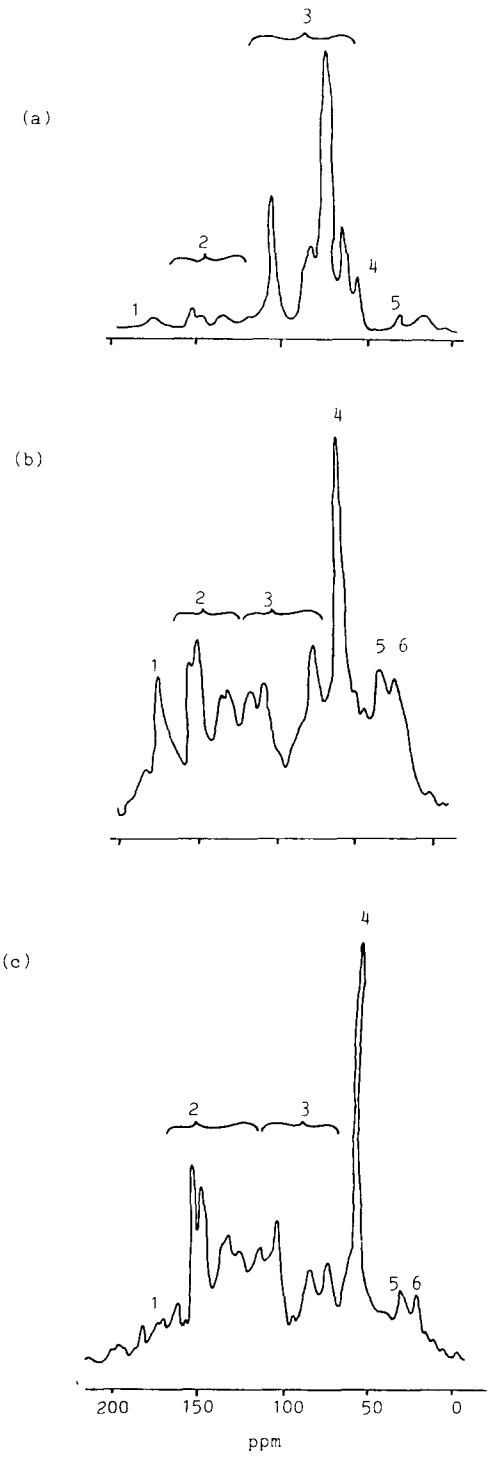

Fig. 2. CP/MAS ^{13}C-NMR spectra of (**a**) ball-milled straw, (**b**) acid-precipitated soluble product of straw degradation by *T. mesophila*, (**c**) milled straw lignin. Peaks and regions are assigned thus: *1*, carbonyl groups of esters/acids of phenylpropanoid and C_α-C_β unsaturated units (175 ppm); *2*, carbons associated with lignin (115—155 ppm); *3*, carbons associated with carbohydrate (65—105 ppm); *4*, methoxyl units on C_3 and C_5 positions of aromatic rings (55 ppm); *5*, saturated carbons (30—45 ppm); *6*, methyl groups (22 ppm)

observed in the spectra presented here (Fig. 2), but is significantly increased in the acid-precipitable actinomycete-solubilised product. The most probable explanation for this is that *T. mesophila* has oxidised the α-carbons in the lignin propanoid chains, a reaction which would be expected to improve lignin solubility. Again it is possible that this is a general feature of actinomycete attack on lignocellulose, since CP/MAS NMR spectra of straw composted for mushroom production contain a similarly intense signal at 175 ppm (unpublished results), and actinomycetes are known to be dominant members of the mushroom compost microflora (Lacey 1973). Furthermore, it has been proposed that in *S. viridosporus*, oxidation of α-carbons is the initial step in the lignin degradation pathway, leading to β-aryl ether cleavage by a mechanism as yet undescribed (Crawford and Crawford 1984). Like *T. mesophila*, *S. viridosporus* forms a soluble acid-precipitable product which is polyphenolic in nature and contains significant amounts of both pentoses and nitrogen (Crawford et al. 1983; Borgmeyer and Crawford 1985). Both organisms are also phenol oxidase negative (unpublished results), in contrast to *S. badius* where phenol oxidase activity is implicated in the polymerisation of low molecular weight intermediates of lignin degradation (Borgmeyer and Crawford 1985).

At this stage it is unclear whether *T. mesophila* produces an enzyme system which specifically attacks intermonomer bonds in lignin. Lignocarbohydrate could be solubilised as the result of a number of reactions including oxidation of lignin side-chains, hydrolysis of xylan and possibly deesterification and demethylation. Cleavage of ester and ether cross links between lignin and carbohydrate could also contribute. Isolation and identification of the enzymes involved is a priority and with appropriate genetic manipulation, could lead to actinomycete-based delignification processes and an improved understanding of natural humification.

Acknowledgements. This work was part of a research programme supported jointly by the British Petroleum Venture Research Unit and the Agriculture and Food Research Council. We are grateful to Edwin Peace for technical assistance and acknowledge the invaluable contribution of Dr. I. J. F. Poplett, British Petroleum Research Centre, Sunbury-on-Thames, Berkshire, England, to the NMR analysis.

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Received February 24, 1986/Revised April 15, 1986