Monitoring bacterial consumption of low molecular weight lignin derivatives by high performance liquid chromatography

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The lignolytic capacity of some natural bacterial isolates was examined. Strains were selected from samples of decaying wood by growth in a minimal medium containing aromatic compounds with a structural relationship to lignin as the sole carbon sources. These included derivatives of benzoic and phenylpropanoic acids, as well as a mixture of low molecular weight compounds obtained by fractionation of kraft lignin. Reversed-phase high-performance liquid chromatography analyses before and after cell growth in the latter revealed a degradation pattern of the different compounds present in the culture which was characteristic for each of the strains studied.

Keywords: Lignin biodegradation; h.p.l.c.; kraft lignin

Introduction

In nature, lignin biodegradation implies a series of reactions which are thought to be carried out mainly by fungi. Increasingly strong evidence, however, points to the participation of bacteria in the dissimilation of this highly abundant biopolymer.[1–5] Moreover, it is becoming widely accepted that fungal–bacterial associations normally operate in the process.

In the laboratory, studies on lignin biodegradation are hindered by the complex tridimensional structure of the macromolecule. The lack of a defined structure of lignin, in addition to the difficulties in obtaining it in a native form, have prompted the use of lignin-derived preparations for these purposes. Among them is kraft lignin, which consists of a mixture of ill-defined compounds that are released after treatment of wood with a solution composed of sodium hydroxide and sodium sulphide.[6] Kraft lignin has proved useful as a carbon source for microbiological studies,[7–10] although it has to be freed of non-lignin contaminants to obtain reliable results.[1] Lundquist *et al.*[11] have reported a procedure that besides eliminating these contaminants, separates kraft lignin in two distinctive fractions: one that is soluble in ether (ESF), whose components possess molecular weights < 3000 and a second one insoluble in ether (EIF), which contains lignin oligomers with molecular weights > 3000.

Since either fraction is heterogeneous with respect to its composition, the actual qualitative and quantitative alterations produced as a consequence of microbial growth in them are unclear. On the other hand, high performance liquid chromatography (h.p.l.c.) has been described as a suitable technique to separate low molecular weight lignins,[12] as well as phenolic compounds.[13] Therefore, in this work we have made use of this methodology to analyse the consumption of the various components present in the ESF of kraft lignin.

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Materials and methods

Chemicals

Vanillic, *p*-coumaric, ferulic, cinnamic and syringic acids, as well as tetrabutylammonium hydrogen sulphate (TBA) were from Sigma Chemical Co., USA. In this work, two sources of kraft lignin were utilized. One of them, indulin ATR-C (Westvaco Corp., North Charleston, USA), was a generous gift from Dr K. Kirk (Madison, WI). The other, Celarauco lignin, was kindly provided by Celulosa Arauco y Constitución (Chile).

Fractionation of lignin

Both indulin and Celarauco lignin were fractionated as described by Lundquist *et al.*[11] Since Celarauco lignin had been supplied as kraft spent liquor, lignin-related oligomers were precipitated from this suspension by acidification to pH 6.0 with hydrochloric acid and collected by centrifugation at 10 400*g* for 20 min before starting the purification procedure.

Isolation and culture of bacterial strains

Samples of soil and rotten wood were suspended in 2.0 ml K salt medium[14] supplemented with trace elements, as described by Mateles *et al.*[15] To this mixture, either ferulic acid or ESF of kraft lignin were added to a final concentration of 5.0 mM or 5.0 mg ml^{-1}, respectively. Stock solutions (10X) of carbon sources had been previously filter sterilized. Cultures were incubated at 30°C in a rotary shaker and, after several transfers, aliquots were serially diluted and plated on agar having the same composition as the above medium. Isolated colonies thus obtained were suspended in 50% glycerol dissolved in K salt medium and were stored at −20°C.

Chromatographic conditions

Reversed-phase h.p.l.c. was carried out on a Waters solvent pumping system (model 6000A, Waters Associates Inc., Milford, MA, USA) attached to a Waters manual injector, model U6K. Detection was at 254 nm utilizing a Waters variable wavelength detector, model 441. A 100 × 8

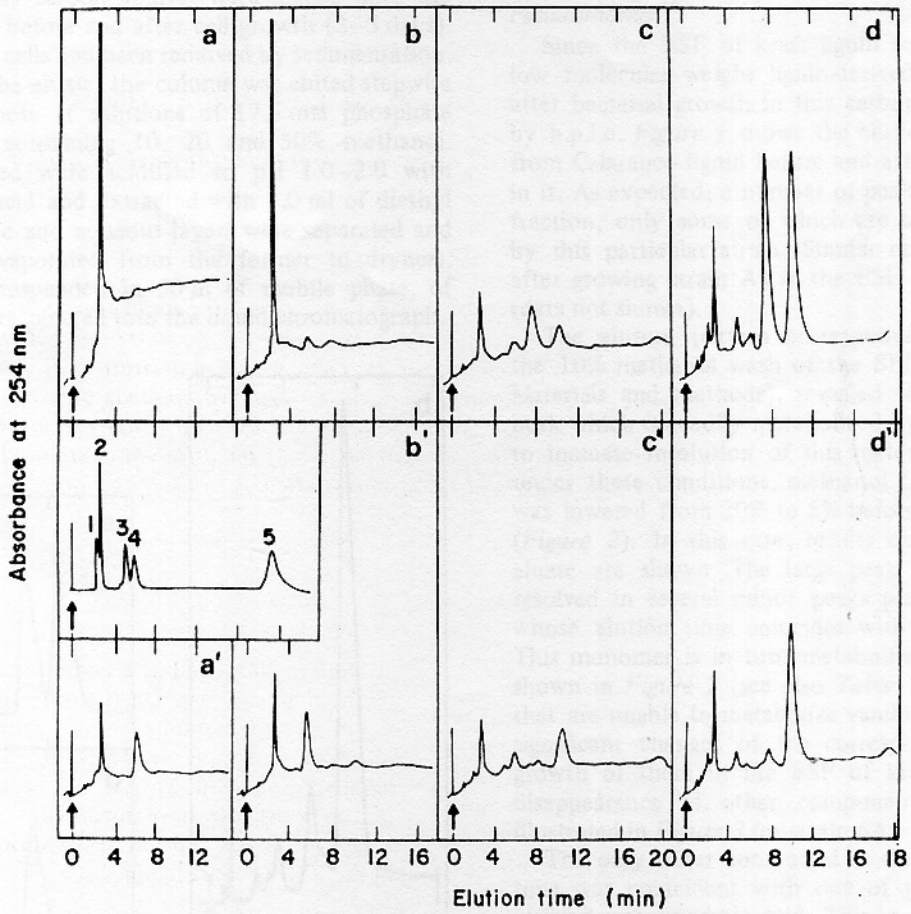

Figure 1 Elution profile of the SEP-PAK precolumn fractions analysed before (a–d) and after (a'–d') growing strain M5 in the ESF of Celarauco lignin. (a, a') Eluate; (b, b') wash with 10% methanol; (c, c') wash with 20% methanol; (d,d') wash with 50% methanol. The insert contains the elution pattern of monomeric standards vanillic (1), syringic (2), *p*-coumaric (3), ferulic (4) and cinnamic (5) acids. The arrows indicate the time of injection of the samples

Table 1 Growth of some natural bacterial isolates on various carbon sources

Strain	Selection media	Growth on				
		Ferulic acid	Vanillic acid	Cinnamic acid	ESF*	EIF*
M5	Ferulic acid	+	+	–	+	–
A1	Ferulic acid	+	+	–	+	–
C2	Ferulic acid	+	+	+	+	–
S10	Ferulic acid	+	+	–	+	–
A6	ESF (indulin)	–	–	–	+	–
S13	ESF (indulin)	+	+	–	+	–
C3	ESF (indulin)	+	–	–	+	+

*From either indulin or Celarauco lignin

mm stainless steel column packed with 10 μm C18 μBondapak was used for chromatographic separations. The mobile phase consisted of 17.5 mM phosphate buffer, pH 7.0, 20% h.p.l.c.-grade methanol (Fisher, USA), 5.0 mM tetrabutylammonium hydrogen sulphate and the flow rate was 3.0 ml min^{-1}. Elution data were recorded with a Servogor 120 BBC Goerz Metrawatt recorder.

In order to improve resolution among the ESF components, samples were previously fractionated in a 1.0 ml SEP-PAK column, packed with the same stationary phase as above. Prefractionation was as follows: aliquots (1.0 ml) of culture media containing ESF of either indulin or Celarauco lignin as carbon source, were loaded onto the SEP-PAK column before and after cell growth (2–3 days). In the latter case, cells had been removed by sedimentation. After collecting the eluate, the column was eluted stepwise with 1.0 ml aliquots of solutions of 17.5 mM phosphate buffer, pH 7.0, containing 10, 20 and 50% methanol. Fractions obtained were acidified to pH 1.0–2.0 with 5 M phosphoric acid and extracted with 1.0 ml of diethyl ether. The organic and aqueous layers were separated and the ether was evaporated from the former to dryness. Samples were resuspended in 50 μl of mobile phase, of which 5–15 μl were injected into the liquid chromatograph.

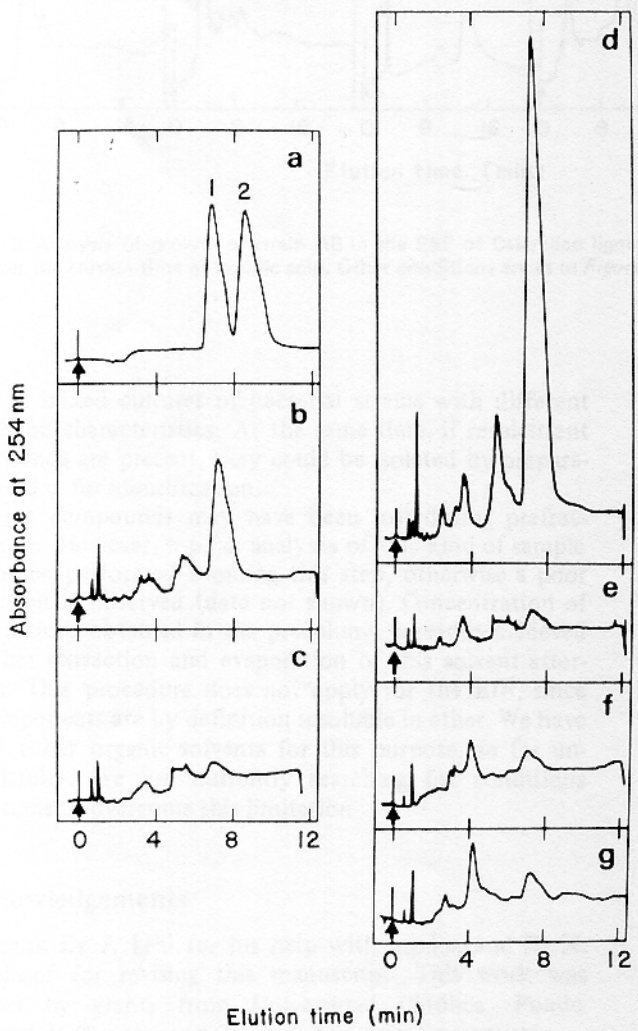

Figure 2 Chromatography of the eluate fractions of the SEP-PAK precolumn utilizing 5% instead of 20% methanol in the mobile phase. (a) Elution pattern of vanillic (1) and syringic (2) acids; (b) ESF from Celarauco lignin; (c) as in b, after growth of strain M5; (d) ESF from indulin; (e–g) as in d, after growth of strains A2, C2 and S10, respectively

Results

About fifty bacterial strains have been isolated following the procedure described in Materials and methods. *Table 1* shows the ability of some of these strains to metabolize different lignin-related carbon sources. Of those selected in ferulic acid, all metabolize vanillic acid and are also able to grow in the ESF of kraft lignin. In turn, selection in the ESF does not imply capacity to metabolize these aromatic monomers. Cinnamic acid and the EIF are only metabolized by strains C2 and C3, respectively. Based on preliminary information obtained concerning the biochemical properties of strains listed in *Table 1*, they all belong to the genus *Pseudomonas*.[16]

Since the ESF of kraft lignin consists of a mixtue of low molecular weight lignin-derived compounds, its fate after bacterial growth in this carbon source was analysed by h.p.l.c. *Figure 1* shows the elution pattern of the ESF from Celarauco lignin before and after growth of strain M5 in it. As expected, a number of peaks were detected in this fraction, only some of which are selectively metabolized by this particular strain. Similar observations were made after growing strain A1 in the ESF obtained from indulin (data not shown).

The elution pattern corresponding to the eluate and the 10% methanol wash of the SEP-PAK precolumn (see Materials and methods), revealed the presence of a large peak which is readily metabolized by both strains. In order to increase resolution of this material which elutes early under these conditions, methanol from the mobile phase was lowered from 20% to 5% before injecting the samples (*Figure 2*). In this case, results obtained only with the eluate are shown. The large peak observed is now well resolved in several minor peaks plus a major component whose elution time coincides with that of vanillic acid. This monomer is in turn metabolized by the four strains shown in *Figure 2* (see also *Table 1*). In contrast, strains that are unable to metabolize vanillic acid do not produce significant changes of the corresponding peak, although growth of them in the ESF of kraft lignin leads to the disappearance of other components. This behaviour is illustrated in *Figure 3* for strain 6A.

The only other compound of the ESF whose retention time was coincident with one of those of the standards utilized was cinnamic acid. This monomer, which has the largest elution time of all five, was found as a minor component in both indulin and Celarauco lignin. *Figure 4* displays the elution pattern of the 50% methanol wash of the SEP-PAK precolumn, before and after growth of strain C2 in the ESF of indulin. This strain, which is the only one able to metabolize cinnamic acid, consumes this monomer quantitatively, in addition to some of the other components of the mixture. Attempts to confirm the identity of these, as well as other compounds, are now under way.

Discussion

According to the evidence presented in this report, h.p.l.c. appears to be an attractive technique to study microbial degradation of kraft lignin. In addition, it is also useful to analyse the composition of kraft lignins from different sources, provided the appropriate standards are available.

One feature of particular interest is that the degradation patterns observed are strain-specific. In this sense, it would be interesting to explore the possibilities of reaching quantitative consumption of the kraft lignin oligomers

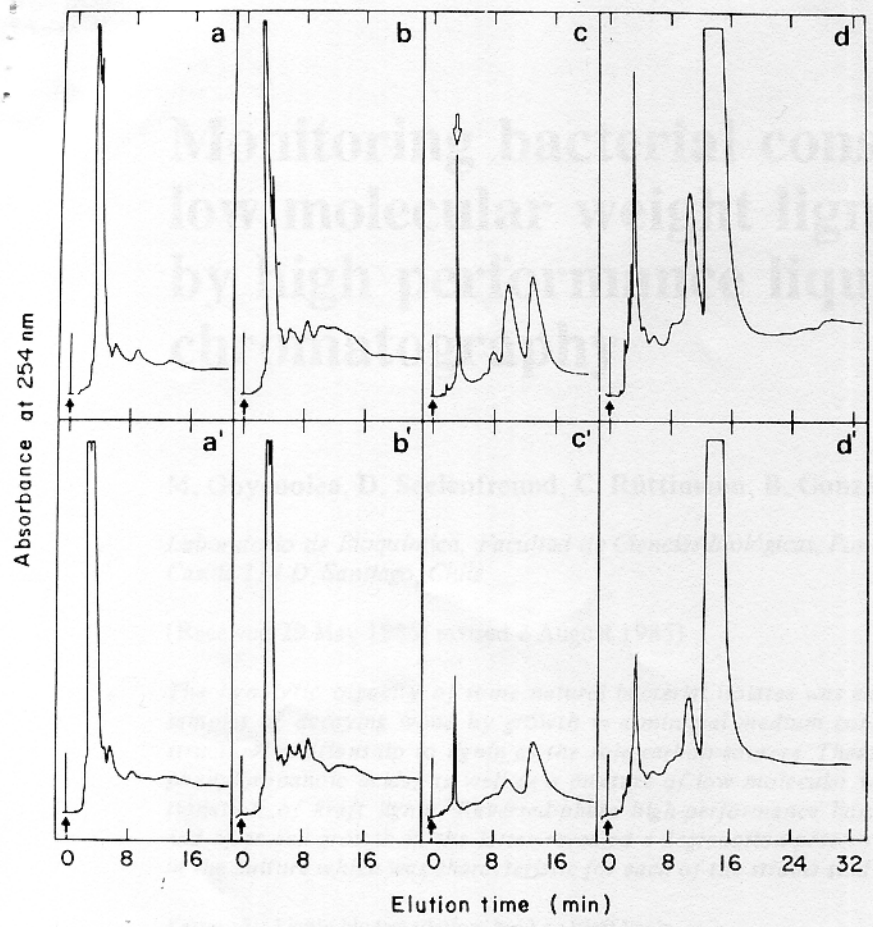

Figure 3 Analysis of growth of strain A6 in the ESF of Celarauco lignin. The open arrow indicates the elution time of vanillic acid. Other conditions are as in *Figure 1*

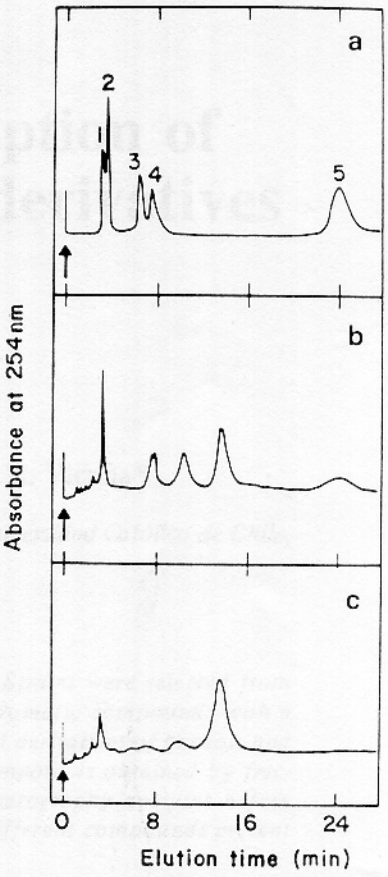

Figure 4 Elution profile of the 50% methanol wash of SEP-PAK precolumn before (b) and after (c) growing strain C2 in the ESF of indulin. (a) Elution pattern of monomeric standards as in *Figure 1*

through mixed cultures of bacterial strains with different metabolic characteristics. At the same time, if recalcitrant compounds are present, they could be isolated by preparative h.p.l.c. for identification.

Some compounds may have been lost during prefractionation. However, h.p.l.c. analyses of this kind of sample cannot be performed avoiding this step, otherwise a poor resolution is observed (data not shown). Concentration of the fractions obtained in the precolumn is readily achieved by ether extraction and evaporation of this solvent afterwards. This procedure does not apply for the EIF, since its components are by definition insoluble in ether. We have tested other organic solvents for this purpose, so far unsuccessfully. We are currently searching for conditions conditions to overcome this limitation.

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References

1 Crawford, R. L. *Lignin Biodegradation and Transformation* John Wiley and Sons, New York, 1981

2 Crawford, R. L. and Crawford, D. L. *Enzyme Microb. Technol.* 1984, 6, 434–442

3 Kirk, T. K. in *The Filamentous Fungi* (Smith, J. E. and Kristiansen, B., eds) Edward Arnold, London, 1983, vol. 4, p. 266–295

4 Kirk, T. K. in *Microbial Degradation of Organic Compounds* (David T. Gibson, ed.) Marcel Dekker, New York 1984, vol 13, pp. 399–437

5 Janshekar, H. and Fiebhter, A. *Adv. Biochem. Eng. Biotechnol.* 1983, 27, 119–179

6 Sjöstrom, E. *Wood Chemistry: Fundamentals and Applications* Academic Press, London, 1981, pp. 104–105

7 Deschamps, A. M., Mahoudeau, G. and Lebeault, M. *Eur. J. Appl. Microbiol. Biotechnol.* 1980, 9, 45–51

8 Ander, P. and Eriksson, K.-E. *Prog. Ind. Microbiol.* 1978, 14, 1–58

9 Lundquist, K., Kirk, T. K. and Connors, W. J. *Arch. Microbiol.* 1977, **112**, 291–296

10 Kerr, T. J., Kerr, R. D. and Benner, R. *Appl. Environ. Microbiol.* 1983, **46**, 1201–1206

11 Lundquist, K. and Kirk, T. K. *Tappi J.* 1980, 63, 80–82

12 Himmel, M. E., Oh, K. K., Sopher, D. W. and Chum, H. L. *J. Chromatogr.* 1983, **267**, 249–265

13 Villeneuve, F., Abravanel, G., Moutounet, M. and Alibert, J. *J. Chromatogr.* 1982, **234**, 131–140

14 Salkinoja-Salonen, M. S., Vaisanen, E. and Paterson, A. in *Plasmids of Medical, Environmental and Commercial Importance* (Timmis, K. N. and Pühler, A., eds) Elsevier–North Holland, Amsterdam, 1979, pp. 301–314

15 Mateles, R. I., Baruah, J. N. and Tannenbaum, S. R. *Science* 1967, **157**, 1322–1323

16 Krieg, N. R. and Holt, G. J. *Bergey's Manual of Systematic Bacteriology* 9th edn, Williams and Wilkins, Baltimore, USA, 1984