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Analysis of alkali-lignin in a paper mill effluent decolourised with two *Streptomyces* strains by gas chromatography–mass spectrometry after cupric oxide degradation☆

Manuel Hernández, María Jesús Hernández-Coronado, Maria Dolores Montiel, Juana Rodríguez, María Enriqueta Arias*

Departamento de Microbiología y Parasitología, Universidad de Alcalá, 28871 Alcalá de Henares (Madrid), Spain

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Abstract

Alkali-lignin samples obtained from an untreated paper mill effluent and from the effluent decolourised by the strains *Streptomyces avermitilis* CECT 3339 and *Streptomyces scabies* UAH 51 were analysed by gas chromatography–mass spectrometry (GC–MS) after cupric oxide degradation. The analysis of the depolymerisation products of the alkali-lignin from the decolourised effluents showed a strain specific modification of the aromatic moiety of the alkali-lignin. Moreover, both strains were able to breakdown the aryl–alkyl ether linkages between the cinnamic acids and the lignin. Finally, GC–MS analysis showed that both strains oxidised the alkali-lignin regardless of its initial degree of oxidation.

Keywords: *Streptomyces* spp.; Paper; Lignin; Alkali-lignin

1. Introduction

To reduce environmental problems recent regulations demand that effluents from pulp and paper industries must be treated before being discharged into continental waters. These brown-coloured effluents, containing highly oxidised compounds and partially degraded lignin, can have a major detrimental effect on the environment [1]. To date, a number of biological processes have been applied to decolourise these effluents. These include the use of ligninolytic microorganisms such as white-rot fungi [2–4] or actinomycetes [5,6].

In previous studies, actinomycetes have proved their usefulness to remove the colour from a dark coloured paper mill effluent obtained after semichemical alkaline pulping of wheat straw [6,7]. In these studies the residual alkali-lignin fraction from the decolourised effluent was analysed by nuclear magnetic resonance (NMR) spectroscopy [7] and pyrolysis coupled to gas chromatography–mass spectrometry (Py–GC–MS) [8]. According to this study, an increase in the degree of oxidation of the lignin,

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*Corresponding author. Tel.: +34-91-8854-633; fax: +34-91-8854-623.

E-mail address: enriqueta.arias@uah.es (M.E. Arias).

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which could be directly correlated with the degradation of this polymer [9], was only detected in the alkali-lignin of the decolourised effluent when ^{13}C-NMR cross polarisation magic angle spinning (CP-MAS) was performed. In contrast, throughout the analysis of the alkali-lignin by Py–GC–MS, an alteration of the aromatic moiety of the lignin was inferred. However, the dramatic depolymerization of lignin caused by this technique precludes the identification of cinnamic acids separately from lignin units.

Because of these difficulties, CuO degradation coupled to GC–MS is considered a suitable technique to analyse lignin from agricultural residues and its derivatives, taking into account the high proportion of cinnamic acids that are present [10–12]. Recently, a modification of the traditional procedure [10] was applied to woody and non-woody materials by using microwave digestion system [13]. The relatively mild conditions under which CuO oxidises lignin, allows the cleavage of β-aryl-ether bonds without alteration of the propyl side-chain of the lignin moiety [14]. After CuO degradation of lignin, nine products corresponding to the three lignin units (*p*-hydroxyphenyl, guaiacyl and syringyl) could be identified. Moreover, the degree of oxidation among these compounds could be distinguished by this technique.

In this work, the alkali-lignin fraction obtained from a paper mill effluent decolourised by two *Streptomyces* strains was analysed by GC–MS after CuO degradation. Differences in the mechanism of lignin attack between these strains can be deduced through the application of this technique.

2. Experimental

2.1. Reagents

The reagents used in this work as well as the source and purity are as follows: Tween 80; HCl 99.9% pure (36.5–38% HCl content); CuO; ethylvanilline (3-methoxyphenylacetophenone); *p*-hydroxybenzaldehyde; *p*-hydroxyacetophenone; *p*-hydroxybenzoic acid; vanillin (4-hydroxy-3-methoxybenzaldehyde); acetovanillone (4-hydroxy-3-methoxyacetophenone); vanillic acid (4-hydroxy-3-methoxybenzoic acid); syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde); acetosyringone (4-hydroxy-3,5-dimethoxyphenylpropanone); syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid); *trans*-ferulic (4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric (*p*-hydroxycinnamic acid) (Sigma–Aldrich, Milwaukee, WI, USA, as ACS reagent grade). Glycerol, purissimum cedex grade; $(NH_4)_2SO_4$, extra pure ACS reagent grade and NaOH extra pure ACS reagent grade (Scharlau, Barcelona, Spain). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 99% pure (Panreac, Barcelona, Spain) and *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) (Merck, Darmstadt, Germany).

2.2. Microorganisms and culture conditions

Two *Streptomyces* strains recently classified as *S. avermitilis* CECT 3339 and *S. scabies* UAH 51 were selected in our laboratory for their ability to remove the colour from a paper mill effluent [6]. The effluent, provided by a Spanish paperboard manufacturer, was obtained after semichemical alkaline pulping of wheat straw (soda-cook liquor) and was subjected to both anaerobic and aerobic treatments. Spore suspensions of the microorganisms stored at −20°C were used to inoculate GAE agar plates [6]. After 5–6 days of growth, spores were harvested with 0.01% (w/v) Tween 80 and 200 μl of a spore suspension containing 10^7 colony forming units (cfu) were used as inoculum. Cultures were grown in 250-ml flasks containing 50 ml mineral salt medium [15] supplemented with 80% (v/v) total effluent, 1% (w/v) glycerol and 0.2% (w/v) ammonium sulfate. Cultures were incubated for 7 days at 37°C for *S. avermitilis* and at 28°C for *S. scabies* with shaking at 200 rpm. Uninoculated effluent containing the same medium was used as control.

2.3. Alkali-lignin preparation

Untreated and decolourised effluents were acidified with 12 *M* HCl to pH 1–2 and then centrifuged at 12 000 *g* for 10 min. The alkali-lignin obtained after centrifugation was washed with deionised water and freeze–dried in a Christ Alpha 1-4

freeze dryer with LDC controller (B. Braun Biotech International).

2.4. Cupric oxide degradation

Alkali-lignin samples (50 mg) were maintained at 180°C for 3 h in a nitrogen atmosphere in PTFE bombs containing CuO (2 g), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (200 mg) and 14 ml boiled 2 *M* NaOH. After precipitation with 12 *M* HCl, degradation products were recovered with three volumes of 20 ml diethyl ether and dried under nitrogen [10]. Pyridine (100 μl) was added to the dried residues and 25 μl derivatised (silylated) with 40 μl BSTFA. The mixture was heated at 60°C for 10 min with periodic shaking to dissolve residues. Chromatographic analyses of alkali-lignin were carried out with a Perkin-Elmer Sigma 3B gas chromatograph (Norwalk, CT, USA), coupled to a Finnigan MAT ion trap detector (San Jose, CA, USA). Trimethylsilyl ether derivatives were separated using a SPB-1 capillary column (0.25 μm stationary phase thickness, 30 m×0.25 mm I.D. from Supelco, Bellefonte, PA, USA). Nitrogen was used as carrier gas. The initial column oven temperature was 100°C; the temperature was increased at 4°C min^{-1} to a final value of 270°C [16].

Quantifications were based on the area of ethylvanilline (3-methoxyphenylacetophenone) as internal standard and the response factors obtained from standard compounds: three *p*-hydroxyphenyl compounds (*p*-hydroxybenzaldehyde, *p*-hydroxyacetophenone, and *p*-hydroxybenzoic acid); three guaiacyl compounds [vanillin (4-hydroxy-3-methoxybenzaldehyde), acetovanillone (4-hydroxy-3-methoxyacetophenone), and vanillic acid (4-hydroxy-3-methoxybenzoic acid)]; and three syringyl compounds [syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), acetosyringone (4-hydroxy-3,5-dimethoxyphenylpropanone), and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)]; *trans*-ferulic (4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric (*p*-hydroxycinnamic acid) acids. All standard compounds were silylated as described earlier. Concentrations of lignin degradation products were calculated by integration of the chromatographic peaks in a Magnum System program, version 2.4 and expressed as molar quantities.

3. Results

Gas chromatograms corresponding to the degradation products obtained after CuO oxidation of the alkali-lignin from untreated effluent and effluent decolourised by *S. avermitilis* and *S. scabies* after 7 days of incubation are shown in Fig. 1. Internal standard (peak IV), products derived from *p*-hydroxyphenyl (H) units (peaks I, II and VI), guaiacyl (4-hydroxy-3-methoxyphenyl) (G) units (peaks III, V and IX), syringyl (4-hydroxy-3,5-dimethoxyphenyl) (S) units (peaks VII, VIII and X) and cinnamic acids (peaks XI and XII) were identified in all samples. Cinnamic acids, *p*-coumaric and ferulic acids, were mainly detected as *trans*-isomers. Although a small amount of *cis*-isomer was also identified, this isomer has not been taken into account for the quantification of the cinnamic acids present in the different samples.

The molar H:G:S relationship and cinnamic acids content are shown in Table 1. The H:G:S relationship from alkali-lignin obtained from untreated effluent indicated that the lignin corresponding to this effluent was mainly composed of S units. However, in the effluent decolourised by both *Streptomyces* strains, an enrichment in H units could be observed compared with the control. Moreover, in alkali-lignin from decolourised effluent by *S. avermitilis*, a decrease in the G units content was detected; in contrast, that obtained from effluent decolourised by *S. scabies* showed a decrease in both G and S units (Table 1).

In all samples, the ferulic acid content was higher than that of *p*-coumaric acid. Although both strains reduced the cinnamic acids content from the alkali-lignin, the most remarkable decrease corresponded to *S. avermitilis*.

As indicated in Table 2, the concentration (μmol/100 mg sample) of aldehyde-, ketone- and acid-type compounds identified in the alkali-lignin obtained from untreated and decolourised effluents varied considerably. Results obtained from control alkali-lignin showed a higher proportion of acid-type compounds than aldehyde and ketone-type compounds. Even though, in the alkali-lignin corresponding to the effluent decolourised by both strains, an increase in the oxidation degree was detected, mainly in that corresponding to *S. scabies*.

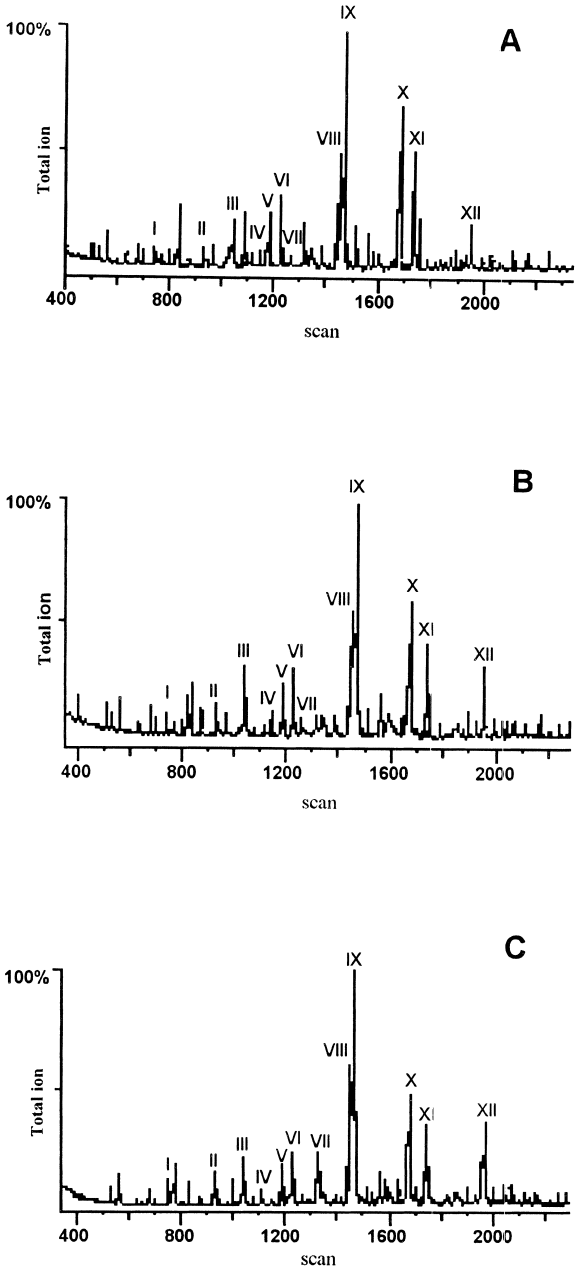

Fig. 1. Gas chromatograms corresponding to the depolymerization products of the alkali-lignin obtained from: untreated effluent (A), effluent decolourised by *S. avermitilis* (B) and *S. scabies* (C), after 7 days of incubation. Chromatographic peaks correspond to: I, *p*-hydroxybenzaldehyde; II, *p*-hydroxyacetophenone; III, vanillin; IV, ethylvanillin (internal standard); V, acetovanillone; VI, *p*-hydroxybenzoic acid; VII, syringaldehyde; VIII, acetosyringone; IX, vanillic acid; X, syringic acid; XI, *p*-coumaric acid; XII, ferulic acid.

4. Discussion

CuO degradation is a suitable technique to carry out quantitative and qualitative analysis of lignin from *Gramineae*. The degradation products can be easily derivatised, separated by GC and identified by MS, allowing for the quantification of lignin units separately from cinnamic acids [14].

The H:G:S relationship for alkali-lignin obtained from uninoculated effluent indicated that lignin present in this fraction is mainly composed of syringyl (3,5-dimethoxy-4-hydroxy phenyl) units. This fact could be explained on the basis of a preferential solubilisation of these units during the alkaline cooking of wheat straw during the pulping process [17]. The higher solubilisation degree of S units must be attributed to the inability of these units to establish linkages in both positions 3 and 5 of the aromatic ring with closer lignin units. Thus, these units are less condensed than guaiacyl and *p*-hydroxyphenyl units which are able to establish different linkages in positions 5, and 3 and 5, respectively. Moreover, in the alkali-lignin from the untreated effluent a high content of H units was detected compared with that corresponding to native lignin in wheat straw [18]. It should be noted that the paper mill effluent used in this study was previously treated with an anaerobic and an aerobic treatment. Thus, the high proportion of H units detected in the decolourised effluent suggests that these units are inaccessible to the *Streptomyces* strains. However, in the alkali-lignin obtained from decolourised effluent, a decrease in S units and in both S and G units was detected in the case of *S. avermitilis* and *S. scabies*, respectively. These results confirm the inability of *Streptomyces* strains to attack the highly condensed units of lignin. However, a strain specific modification in S and/or G units of the aromatic moiety of lignin during the decolourisation process was evident.

Cinnamic acids, which are involved in linking lignin and hemicellulose fractions of lignocellulose of Herbaceae, were detected in the alkali-lignin fraction. *p*-Coumaric (4-hydroxycinnamic acid) and ferulic (3-methoxy-4-hydroxycinnamic acid) acids are bifunctional; they are able to form ester and ether linkages by reaction of their carboxyl or phenolic groups, respectively [19]. During the alkaline ex-

Table 1
Concentration (μmol per 100 mg sample) of the different lignin units, molar H:G:S relationship, and cinnamic acids content determined by CuO degradation of the alkali-lignin (AL) obtained from untreated effluent (control) and effluent decolourised by *S. avermitilis* and *S. scabies*

	Concentration (units)			Molar ratio, H:G:S	Cinnamic acid content (units)	
	H	G	S		*p*-Coumaric	Ferulic
AL-control	0.96	3.31	4.2	11:39:50	2.08	4.34
AL-*S. avermitilis*	2.03	3.32	3.99	22:35:43	1.57	3.15
AL-*S. scabies*	1.61	2.7	3.83	20:33:47	1.99	3.73

traction of the pulping process most of the ester linkages are broken, but some cinnamic acids still remain bound to the lignin by ether linkages.

GC–MS analysis of the depolymerization products of alkali-lignin from untreated effluent showed a doubled proportion of ferulic acid compared with *p*-coumaric acid. This difference could be due to the fact that ferulic acid is mainly linked to hemicellulose through ester bonds and with lignin through ether linkages [11,12]. However, the small amount of *p*-coumaric acid would correspond to that linked to lignin by ether bonds [20,21].

Results corresponding to alkali-lignin obtained from decolourised effluent also showed that ferulic acid was in a higher proportion than *p*-coumaric acid although both strains were able to reduce the content of these compounds in the residual lignin. It must be emphasised that *S. avermitilis* reduced the content of cinnamic acids to a greater extent than *S. scabies*. This result could be a consequence of the action of oxidative extracellular enzymes able to break aryl–alkyl ether linkages. In fact, peroxidases have been previously reported in this strain [22] and also have been widely described in other actinomycetes [23–25].

Finally, through the analysis of the depolymerisation products obtained after CuO degradation, the oxidation degree of alkali-lignin from both untreated and decolourised effluent was determined. Traditionally, the increase in the degree of oxidation of lignin is considered a parameter directly related to the extent of degradation of this polymer. An increase in the degree of oxidation has been observed in lignocellulosic residues degraded by fungi [9] as well as in other materials transformed by actinomycetes [26–28].

Analysis of degradation products derived from control alkali-lignin showed a higher proportion of acid-type compounds than aldehyde or ketone type. These data also demonstrated that the lignin present in the effluent was partially degraded during the anaerobic and aerobic treatment carried out in the industry. In contrast, data obtained with CuO degradation of native lignin from different vegetal tissues showed that aldehyde-type compounds were always more abundant than ketone- or acid-type compounds [10]. Results from the analysis of alkali-lignin obtained from decolourised effluent showed that both strains increased the oxidation degree of lignin compared with the control. This result confirms the high oxidative ability of streptomycetes on lignin moiety, regardless of its initial degree of oxidation. These microorganisms are thus extremely useful for application involving clean technologies and for biotechnological purposes in which the oxidation of recalcitrant molecules such as lignin is mandatory.

Table 2
Concentration (μmol per 100 mg sample) of acid-, aldehyde- and ketone-type compounds and ratio AC/(CE+KE) obtained from alkali-lignin from untreated and decolourised effluents

	Acid	Aldehyde	Ketone	AC/(AL+KE)[a]
AL-control	3.71	2.5	2.26	0.77
AL-*S. avermitilis*	4.41	1.9	3.04	0.89
AL-*S. scabies*	5.05	0.95	2.15	1.63

[a] Acid/(aldehyde+ketone).

In conclusion, *S. avermitilis* and *S. scabies* proved their utility in degrading oxidised and highly condensed lignin present in the paper mill effluent. This degradation could be considered strain specific although both strains were able to modify the aromatic moiety of the lignin and to break the aryl-alkyl ether bonds between the lignin and cinnamic acids.

The use of CuO degradation coupled to GC–MS was found to be a suitable technique for providing novel and useful information about the biological action of *Streptomyces* on wheat straw lignin present in an alkaline effluent derived from the paper industry.

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