Biodegradation **12:** 219–223, 2001.

Degradation of alkali-lignin residues from solid-state fermentation of wheat straw by streptomycetes

Manuel Hernández[1], María J. Hernández-Coronado[1], Andrew S. Ball[2] & María E. Arias[1]*
[1]*Departamento de Microbiologia y Parasitologia, Universidad de Alcala, 28871 Alcalá de Henares, Spain;* [2]*Department of Biological Sciences. University of Essex, Wivenhoe Park, Colchester C04 3SQ, U.K. (*author for correspondence)*

Key words: Alkali-lignin, decolourisation, degradation, *Streptomyces*

Abstract

The ability of three *Streptomyces* strains to degrade alkali-lignin, produced from the treatment of wheat straw by the same organisms, was examined. Decolourisation and loss of alkali-lignin was only detected in cultures supplemented with ammonium as an inorganic N source. The pH of cultures supplemented with inorganic N reached lower pH than in those supplemented with yeast extract. From FT-IR spectra corresponding to the alkali-lignin obtained from the same cultures, a degradation of carbohydrate component concomitant with a modification in the aromatic moiety of lignin could be inferred. The results indicate that streptomycetes are suitable for use in the treatment of alkali-lignin effluents from the biological treatment of wheat straw by the same organisms and therefore support the role for these organisms in the development of clean technologies in pulp and paper industry.

Introduction

There currently exists great interest in developed countries for the substitution, either totally or partially, of chemical methods used in the paper-mill industries by biological methods (Archibald et al. 1997; Viikari et al. 1994). The reasons for this interest are two-fold; firstly, biological methods may result in energy-cost savings, and secondly, may result in a reduction in the environmental impact of the paper and pulp industry (Joyce et al. 1993; Messner & Srebotnik 1994). These biological methods are based on the ability of different groups of micro-organisms, or their enzymes to transform lignocellulosic residues from different origins. Wheat straw an other grass residues are considered a good alternative to wood for pulp and paper industry (Giovannozzi-Sermanni et al., 1994; Sabharwal et al. 1994; Guadalix et al. 1996). However, the brown coloured effluents produced during the pulping process of these lignocellulosic materials also caused serious damage to aquatic environments (Shimp & Owens 1993).

Streptomycetes represent one such group of organisms on the basis of their degradative ability on lignocellulosic residues through the production of a wide array of extracellular oxidative and hydrolytic enzymes in different conditions (Trigo & Ball 1994; Berrocal et al. 1997). Streptomycetes therefore, offer considerable potential for use as a biological alternative in processes related with pulp and paper industry.

However, what is not yet clear, and a factor preventing the development of this biotechnology is whether the use of biological methods in pulp production from these residues will also result in the production of highly coloured effluents which have a large environmental impact and which may be difficult to degrade. Again, streptomycetes can be seen as potentially useful organisms as they are capable not only of solubilising lignin – resulting in an acid-precipitable polymeric lignin named APPL –, but also of decolourising and degrading industrial paper-mill effluents (Hernández et al. 1994). Previously, we have reported the ability of streptomycetes to facilitate the lignin extraction when an alkaline treatment was applied to the fermented wheat straw in comparison

with uninoculated substrate (Hernández-Coronado et al. 1997). Clearly, it would be an advantage if the same organism could carry out delignification and degradation of the subsequent alkali-lignin. Here we report the ability of *Streptomyces* to decolourise and degrade the alkali-lignin produced during this pretreatment.

Materials and methods

Strains and growth conditions

Three *Streptomyces* strains (*S*. UAH 23, *S*. UAH 33 and *S*. UAH 52) were isolated in our laboratory and selected for their ability to degrade lignocellulosic residues (Hernández-Coronado et al. 1997). *S*. UAH 23 was previously classified as *S. chattanoogensis* CECT 3336 (López-Fernandez et al. 1995) and *S*. UAH 33 and *S*. UAH 52 were recently classified through the analysis of whole cell fatty acids by gas chromatography (Microbial ID, Inc.) as *S. violaceus* CECT 3350 and *S. cyaneus* CECT 3351 respectively. Spore suspensions were obtained by growing the strains at 28 or 37 °C (depending on the strain) on GAE agar (Hernández et al. 1994). The suspensions were kept at −20 °C in 20% (wt/vol) glycerol. For the experiments, stock cultures were inoculated onto the same medium, and the spores were harvested with distilled water. Standard spore suspension (10^7 cfu ml^{-1}) were used as initial inocula in all assays.

Production of alkali-lignin

Alkali-lignin was extracted from wheat straw (10 g) transformed by selected *Streptomyces* strains after 14 days incubation in solid-state conditions (Hernández-Coronado et al. 1997). To obtain alkali-lignin, 40 ml of NaOH (0.1 M) was added to transformed wheat straw and steamed at 100 °C for 1 h, then filtered through Whatman No. 54 filter paper and washed again with 400 ml NaOH at 80 °C.

Inocula and culture conditions

Cultures were grown in 100 ml flasks containing 20 ml of 80% (vol/vol) alkali-lignin solution (corresponding to 0.12 mg dry weight of alkali-lignin) containing mineral salt medium (Hernández-Coronado et al. 1997) supplemented with yeast extract (0.6% wt/vol) or glycerol (1% wt/vol) and ammonium sulphate (0.2% wt/vol). The alkali-lignin was extracted from wheat straw transformed by each of the three selected strains. A volume of the standard spore suspension (200 μl) was used to inoculate the medium with the corresponding alkali- lignin so that the organism which was inoculated was the organism used for the production of alkali-lignin in that flask. The initial pH of each medium was adjusted to 7.0 using HCl (12 M). Cultures were incubated for 7 days at 28 °C or 37 °C as appropriate with shaking at 180 rpm. As a control, uninoculated medium was used in all cases.

Cell growth

Cell growth was estimated by determining the dry weight of the mycelia. Following separation of the mycelia from the media by centrifugation at 3000 × *g* for 10 min, the mycelia were dried at 60 °C until a constant dry weight was achieved. Cell growth was expressed as mg dry weight per ml.

Measurement of colour loss and alkali-lignin content

Following removal of mycelia, the colour intensity of culture supernatants was measured at 465 nm, after adjusting the pH of the supernatant to 7.6 using 12 M HCl. The amount of colour present was determined spectrophotometrically and was related to the absorbance of a Pt-Co standard solution at the same wavelength (Hernández et al. 1994). The adsorption of colour by the mycelia was estimated following the addition of NaOH (1M) to the mycelial pellet. Then, suspension was filtered through Whatman No. 1 filter paper and colour content was estimated in the resultant filtrate as described above.

Content of residual alkali-lignin

Untreated and decolourised supernatants were acidified with 12 M HCl to pH 1–2 and then centrifuged at 12000 × *g* for 10 min. Residual alkali-lignin was obtained after each precipitate had been washed with deionized water and freeze-dried in a Christ Alpha 1–4 freeze dryer with an LDC-1 M controller (B. Braun Biotech). Alkali-lignin loss (%) in the supernatants decolourised by the strains was determined daily as dry weight (estimating 100% as the alkali-lignin present in the same volume of uninoculated medium).

IR spectroscopy

FT-IR spectra were carried out with alkali-lignin controls and residual alkali-lignin obtained after five days of incubation for strains *S. chattanoogensis* and *S.*

cyaneus and after seven days of incubation for *S. violaceus*, from the medium containing glycerol and ammonium sulphate. Samples of 3 mg were homogenised with KBr (297 mg) and the disks analysed in a FT-IR Spectrum 2000 spectrophotometer (Perkin Elmer). The spectra were recorded between 400 and 2000 cm^{-1}.

Results and discussion

Decolourisation and removal of alkali-lignin during growth of streptomycetes

The effects of the growth of each of the three streptomycetes on the loss of colour and alkali-lignin loss is shown in Figure 1. When incubated in medium containing glycerol and ammonium sulphate (as C and N source respectively), all three organisms were capable of both decolourising and degrading the alkali-lignin which originated from the same strain (Figure 1A). In all cases greatest loss occurred during the exponential phase of growth. The most significant loss of colour (77% in 5 d) and alkali-lignin loss (90% after 5 d) was detected in medium inoculated with *S. chattanoogensis* (Figure 1A.I). In medium supplemented with yeast extract as the main C and N source only a 20% colour loss was estimated during the exponential phase of growth of all three (Figure 1B). This small decrease was probably attributable to the adsorption of the lignin to the mycelia which was estimated to account for approximately 20% of the loss of alkali-lignin in cultures. These results are in accordance with those obtained when an industrial alkaline paper-mill effluent was used (Hernández et al. 1994). In this study, we demonstrated that cultures containing glycerol and ammonium sulphate resulted in the highest decolourisation of the effluent. In contrast, cultures containing yeast extract resulted in low decolourisation rates. It had previously been reported that solubilisation of lignin occurred preferentially in media supplemented with organic nitrogen, such as yeast extract or amino acids (Iyo & Antai 1988). The results from this study show that the degradation of lignin was more effective in the presence of an inorganic N source, perhaps indicating that preference of inorganic or organic N sources is a variable trait amongst streptomycetes. In fact, an increase in the colour was observed in cultures supplemented with yeast extract. This was attributable to the production of a brown pigment by the three strains in this media,

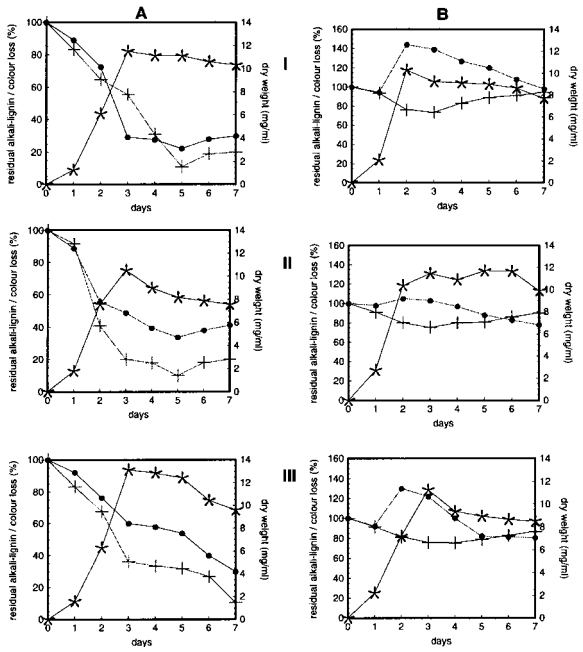

Figure 1. Percentage of residual alkali-lignin (+) and colour loss (●) in medium containing alkali-lignin and either glycerol and ammonium sulphate (A) or yeast extract (B) during the growth (⋆ mg dry weight) of strains *S. chattanoogensis* (1), *S. violaceus* (II) and *S. cyaneus* (III). The results represent the mean of 3 replicates with standard errors within 5% of the mean value presented (100% residual alkali-lignin corresponds to 6 mg l^{-1}).

more remarkable in the case of *S. chattanoogensis* and *S. cyaneus*.

Changes in pH during growth of streptomycetes

To investigate the possible explanation for these results the pH of the culture medium was monitored during growth of three streptomycetes in media containing alkali-lignin supplemented with either inorganic (ammonium sulphate) or organic (yeast extract) N sources (Figure 2). In media supplemented with the inorganic nutrient, a decrease in the pH of the culture medium of all three streptomycetes was observed (Figure 2A), reaching a pH of 5 after 3 days of incubation. In media supplemented with yeast extract, un increase in the pH was observed after 2 days growth, reaching a pH of around 8.5 for all three strains examined (Figure 2B).

To examine whether the loss of colour was merely due to changes in the pH of the culture medium, the effects of the pH of the medium on colour loss was determined in uninoculated medium. No significant loss in colour could be detected in media supplemented with either inorganic or organic nutrients, between

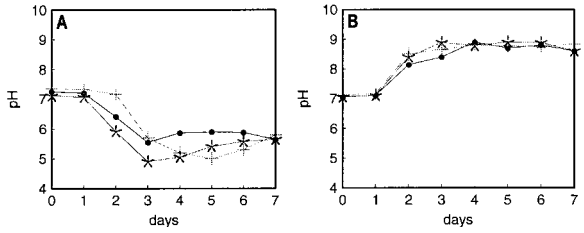

Figure 2. pH values over the time in medium containing alkali-lignin and either glycerol and ammonium sulphate (A) or yeast extract (B), during the growth of strains *S. chattanoogensis* (+), *S. violaceus* (⋆) and *S. cyaneus* (●). The results represent the mean of 3 replicates with standard errors within 5% of the mean value presented.

a pH range of 5 to 9 (data not shown). At a pH value of 4, a reduction in colour of approximately 20% was detected. At pH values below 3, precipitation of alkali-lignin occurred. These results confirm that the decolorization that occurred in media containing alkali-lignin and ammonium sulphate could not be explained in terms of the acidification of the medium but rather as a result of biological degradation of the alkali-lignin. Moreover, previous observations have demonstrated that mineralization of lignin in solid-substrate fermentation preferentially occurred at lower pH values than solubilisation (Pometto III & Crawford 1986).

Spectroscopic study

FT-IR spectra of the controls and residual alkali-lignin corresponding to each strain are shown in Figure 3. In this figure, residual alkali-lignin spectra correspond to the day of incubation for which a maximum decrease in the alkali-lignin content was achieved by accordance with previous studies (Xiao-An et al. 1989; Fidalgo et al. 1993). The spectra obtained from alkali-lignin controls present a similar profile corresponding to the most prominent bands in these spectra to lignin (1510, 1460, 1420, and 1335 cm^{-1}), carbohydrates (1045 and 1110 cm^{-1}), and carbonyl groups (1720 cm^{-1}) (Figure 3.I, III and V). From these spectra it is difficult to establish differences among the strains solubilising lignin from wheat straw. This fact was attributed to the dramatic effect of NaOH used for obtaining lignin from the transformed substrate (Hernández-Coronado et al. 1997). However, in the spectra corresponding to the residual alkali-lignin from each culture, a decrease in the carbohydrates bands was clearly observed along with minor modifications in lignin characteristic peaks. Thus, the decrease in the intensity of the signal at 1045 cm^{-1} corresponded to a decrease in the xylan content (Mansfield et al. 1997) in the spectra obtained from residual alkali-lignin from strains *S. chattanoogensis* (Figure 3.II) and *S. cyaneus* (Figure 3.IV). This result can be attributed to the present of xylanases in the culture media, an observation previously reported in the strain *S. chattanoogensis* (López-Fernández et al. 1995). In the bands corresponding to lignin, a significant decrease in the band intensity at 1420–1400 cm^{-1} was observed in the spectra corresponding to the strains *S. chatianoogensis* and *S. cyaneus*. This decrease in band intensity may be due to aromatic skeletal vibrations (Fidalgo et al. 1993). These results can be explained on the basis of the wide pool of enzymes associated with lignocellulose degradation by *Streptomyces*. Previous studies have demonstrated that these microorganisms produce, under different conditions, oxidative enzymes such as peroxidases (Rob et al. 1997) and laccase-type phenol oxidases (Berrocal et al. 1997) which could be involved in lignin degradation. In addition, hemicellulases (López-Fernandez et al. 1995) and cinnamic acid esterases such as ferulic acid esterases (García et al. 1998) have also been described in streptomycetes and could in some way contribute to the degradation observed in the main components of alkali-lignin.

Conclusions

From this study, it could be concluded that streptomycetes are not only able to decolourise and decrease the alkali-lignin content of culture media containing inorganic N source, but also to modify the lignin component, mainly in the aromatic moiety. This work demonstrates that lignin-carbohydrate complexes, solubilized from wheat straw by *Streptomyces* can be further degraded as long as the metabolism of the nitrogen source of the culture media do not modify the pH up to alkaline values. Related to this, selection of appropriate nutrients (i.e., nitrogen source) may show up the degradative action of these micro-organisms on solubilised lignin from wheat straw.

Further, the observation that the same organism can be used to transform wheat straw lignocellulose and to treat the resulting alkali-lignin effluent, supports the use of biological conversions of these materials.

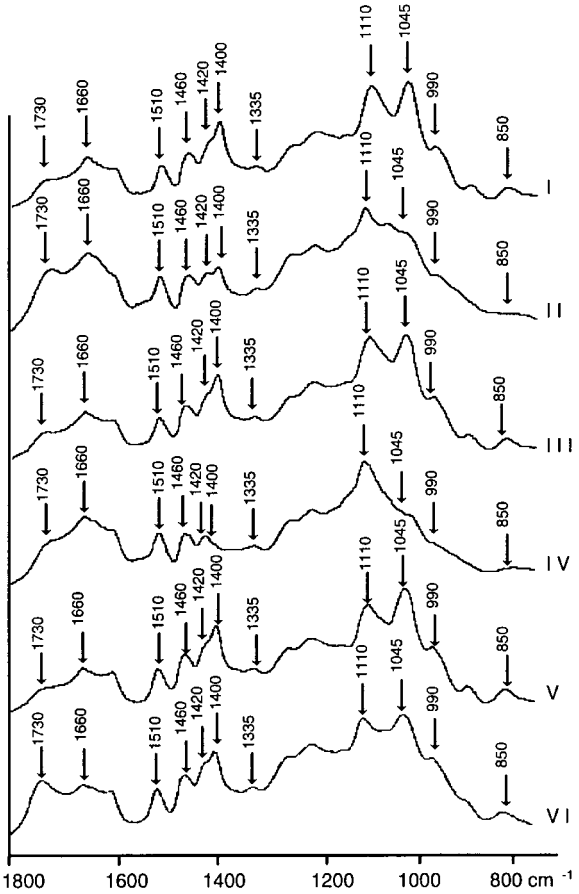

Figure 3. FT-IR spectra corresponding to alkali-lignin controls (I, III, and V) and residual alkali-lignin from cultures of *S. chattanoogensis* and *S. violaceus* after 5 days of incubation (II and VI respectively) and *S. cyaneus* after 7 days of incubation (TV).

Acknowledgements

This work was supported by the Spanish CICYT Project B1097-0808.

References

Archibald FS, Bourbonnais R, Jurasek L, Paice MG & Reid ID (1997) Kraft pulp bleaching and delignification by *Trametes versicolor*. J. Biotechnol. 53: 215–236

Berrocal M, Rodríguez J, Ball AS, Pérez-Leblic MI & Arias ME (1997) Solubilisation and mineralisation of [^{14}C] lignocellulose from wheat straw by *Streptomyces cyaneus* CECT 3335 during growth in solid state fermentation. Appl. Microbiol. Biotechnol. 48: 379–384

Fidalgo ML, Terrón MC, González AF, Martínez AT, González-Vila FJ & Galletti, GC (1993) Comparative studies of fractions from alkaline extraction of wheat straw through chemical degradation, analytical pyrolysis and spectroscopic techniques. J. Agric. Food Chem. 41: 1621–1626

García BL, Ball AS, Rodriguez J, Pérez-Leblic MI, Arias ME & Copa-Patiño JL (1998) Induction of ferulic acid esterase and xylanase activities in *Streptomyces avermitilis* UAH 30. FEMS Microbiol. Lett. 158: 95–99

Giovannozzi-Sermanni G, D'Annibale A, Perani C, Porri A, Pastina F, Minelli V & Gelsomino A (1994) Characteristics of paper handsheets after combined biological pretreatments and conventional pulping of wheat straw. Tappi Journal 77: 151–157

Guadalix ME, Almendros G, Martinez AT, Camarero S, Barrasa JM & Pelayo M (1996) Comparative analysis of wheat straw paperboards prepared after biomechanical and semichemical pulping. Bioresource Technology 57: 217–227

Hernández M, Rodríguez J, Soliveri J, Copa JL, Pérez MI & Arias MF (1994) Paper mill effluent decolorization by fifty *Streptomyces* strains. Appl. Environ. Microbiol. 60: 3909–3913

Hernández-Coronado MJ, Hernández M, Centenera F, Pérez MI, Ball AS & Arias ME (1997) Chemical characterization and spectroscopic analysis of the solubilization products from wheat straw produced by *Streptomyces* strains grown in solid-state fermentation. Microbiology 143: 1359–1367

Iyo AH & Antai SP (1988) Effects of different nitrogen sources on lignocellulose degradation and APPL production. Lett. Appl. Microbiol. 7: 75–78

Joyce TW, Chang H, Campbell AG, Gerad ED & Kirk TK (1983) A continuous biological process to decolorize bleach plant effluents. In Proceedings of Biotechnology in the Pulp and Paper Industry. Sep. 12–14. London.

López-Fernandez CL, Rodriguez J, Copa-Patiño JL, Pérez-Leblic MI & Arias ME (1995) The effects of culture media on the production of xylan-degrading enzymes by *Streptomyces chattanoogensis* UAH 23. J. Basic Microbiol. 35: 405–412

Mansfield SD, de Jong E, Stephens RS & Saddler JN (1997) Physical characterization of enzymatically modified Kraft pulp fibers. J. Biotechnol. 57, 205–216

Messner K & Srebotnik E (1994) Biopulping – An overview of developments in an environmentally safe paper-making technology. FEMS Microbiol. Rev. 13: 351–364

Pometto III AL & Crawford DL (1986) Effects of pH on lignin and cellulose degradation by *Streptomyces viridosporus*. Appl. Environ. Microbiol. 52: 246–250

Rob A, Hernández M, Ball A, Tuncer M, Arias ME & Wilson MT (1997) Production and partial characterization of extracellular peroxidases produced by *Streptomyces avermitilis* UAH 30. Appl. Biochem. Biotechnol. 62: 159–172

Sabharwal HS, Akhtar M, Blanchette RA & Young RA (1904) Biomechanical pulping of kenaf. Tappi Journal 77: 105–112

Shimp RJ & Owens JW (1993) Pulp and paper technologies and improvements in environmental emissions to aquatic environments. Toxicol. Environ. Chem. 40: 213–233

Trigo C & Ball AS (1994) Production of extracellular enzymes during the solubilization of straw by *Thermomonospora fusca* BD25. Appl. Microbiol. Biotechnol. 41: 366–372

Vikari L, Kantelinen A, Sundquist J & Linko M (1994) Xylanases in bleaching: From an idea to the industry. FEMS Microbiol. Rev. 13: 335–350.

Xiao-An L, Zhong-Zhang L & Die-Shang T (1989) Fractional studies on the characteristics of high alkali-soluble lignins of wheat straw. Cellulose Chem. Technol. 23: 559–572