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Effect of *Streptomyces viridosporus* T7A on kraft lignin

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SUMMARY

The ability of the lignino-cellulolytic actinomycete *Streptomyces viridosporus* T7A to attack purified fractions of kraft lignin was examined. In the presence of 0.3% yeast extract, high-molecular weight kraft lignin (MW > 3000, ether-insoluble fraction) does not affect growth of this microorganism significantly, whereas low-molecular weight kraft lignin (MW < 3000, ether-soluble fraction) inhibits its development. Accordingly, average molecular weight of the ether-insoluble fraction after bacterial growth remained unaltered, as measured by Sephadex G-50 gel permeation chromatography. Slight modifications were detected by high performance liquid chromatography in the ether-soluble fraction after incubation with the microorganism. *S. viridosporus* T7A partially decolorized Remazol Brilliant Blue R during growth on wheat lignocellulose. However, decolorization of either fraction of kraft lignin was not observed. These results suggest that the filamentous bacterium *S. viridosporus* T7A is not suitable for pulp mill effluent treatment.

INTRODUCTION

The kraft process is the most common chemical pulping procedure used today [24]. Basically, it involves the cooking of wood chips with a solution of sodium hydroxide and sodium sulfide at 155–175°C. The main delignification reactions taking place under these conditions are cleavage of aryl-alkyl ether bonds, modification of aliphatic side chains and various types of condensations, leading to a dark suspension containing the so called kraft lignin [6,16,24]. In some mills, this mixture is concentrated and used as a fuel, whereas in others it is eliminated through the effluents.

Some filamentous fungi have the capacity to metabolize kraft lignin [11,15]. Based on this property, procedures have been developed for biodecoloration of effluents [9,18]. Researchers have also studied the ability of bacteria [8,14,17] and cultures of mixed microflora [3] to degrade this chemically

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modified lignin. In most cases, degradation has been found to be slow and not extensive. Unexpectedly, kraft lignin is significantly decomposed in the absence of oxygen, under conditions where other lignin preparations are not mineralized [27].

In spite of being frequently utilized in lignin biodegradation studies, purified kraft lignin has not been tested as a substrate with actinomycetes strains. These microorganisms, among them *Streptomyces viridosporus* T7A [1], attack grass lignocelluloses releasing soluble lignin fragments of molecular weight 5 000–20 000 [5,20,21]. This ability to preferentially solubilize rather than mineralize lignin is a property shared by various actinomycetes [21]. The biochemistry of this process is still unclear, although the participation of cellulases has been suggested [7,20,22]. Recently, several isoenzymes of an inducible, extracellular peroxidase have been discovered in cultures of *S. viridosporus* [22], although evidence for cleavage of linkages that are typical of lignin by these enzymes has not yet been provided. Actinomycetes are also able to metabolize small molecular weight lignin substructures, mainly those with a single aromatic ring [23,25].

We decided to study the action of the actinomycete *S. viridosporus* T7A on kraft lignin, since basic knowledge on this subject is lacking and information obtained could lead to practical applications.

MATERIALS AND METHODS

Chemicals

Cinnamic, *p*-coumaric, ferulic, vanillic acids and Remazol Brilliant Blue R were from Sigma Chemical Co., U.S.A. Kraft lignin was kindly provided by the chilean pulping industry Celulosa Arauco y Constitución.

Fractionation of lignin

Kraft lignin was supplied in the form of spent black liquor. In order to separate the lignin-derived components from contaminants present in the suspension, the following steps were carried out: for every 150 ml of spent liquor, 300 ml of water and 32 ml of chloroform were added [26]. The mixture was acidified to pH 1.0–1.5 with concentrated hydrochloric acid under constant agitation and the precipitate obtained was collected by filtration under vacuum and dried. Starting from 150 ml of kraft spent liquor, approximately 20 g of solids were obtained. Purification of this material was then carried out as described by Lundquist *et al.* [19], which results in the fractionation of kraft lignin into an ether-soluble fraction (ESF, MW $<$ 3000) and an ether-insoluble fraction (EIF, MW $>$ 3000). Aqueous stock solutions of ESF and EIF (25 mg/ml) were adjusted with 1.0 N NaOH to pH 11.3 and 9.0, respectively. ESF solutions were filtered through Whatman 1 paper to separate remaining solids (less than 5.0% of the previously weighted material). Both ESF and EIF solutions were filter-sterilized before addition to the culture media.

Preparation of lignocellulose

Ground wheat straw (0.25–2.0 mm) was subjected to successive toluene-ethanol, ethanol and water extractions and then dried at 50°C for twelve hours [2].

Microorganism and culture conditions

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

Cultures of this microorganism were prepared in the salt media previously described [4], containing yeast extract and kraft lignin as indicated in the text. These were inoculated either with spores or mycelia. In the former case, 1.0 ml of a turbid (OD_{600} = 0.3–0.5) suspension of spores in sterile water was added directly to 9.0 ml of the growth medium. Mycelia to be used as inoculum were obtained by adding 1.0 ml aliquots of the spore suspension in water to 9.0 ml of the above salt media containing 0.3% of yeast extract. Incubation at 37°C with vigorous agitation for 24–48 h produced abundant mycelia, which were transferred to the cultures utilizing sterile Pasteur pipettes with cut-off ends.

Cultures in the presence of lignocellulose were prepared as follows: 0.2 ml of the spore suspension were transferred into 250-ml Erlenmeyer flasks containing 200 mg of sterile wheat straw lignocellulose

and 1.8 ml of 0.3% of yeast extract dissolved in culture medium. Stationary incubations were carried out at 37°C for 96 h. At this time, 20 ml of the culture medium supplemented with 0.3% of yeast extract and either 0.05% of the dye or 2.5 mg/ml of the lignin fractions were added to grown mycelia. The flasks were further incubated, now with agitation, for another 25 days.

One ml aliquots were withdrawn at various time intervals for spectrophotometric analysis.

Estimation of biomass

Mycelial growth of *S. viridosporus* T7A was determined by dry weight. Cultures were filtered through pre-weighted G/F glass fiber membranes and dried at 80°C until constant weight. Filtered mycelia exhibited the same color after growth either in the presence or in the absence of kraft lignin, indicating that the latter did not adsorb to the cell surface.

Analytical procedures

Gel permeation chromatography. Filtration of ESF and EIF was carried out in a Sephadex G-50 column of 70 cm × 1.3 cm^2. The column was eluted at a flow rate of 10 ml/h with a solution containing 0.1 N NaOH and 0.1 N LiCl. Blue dextran (MW = 2 × 10^3 kDa), ovoalbumin (45 kDa), chymotrypsinogen A (24 kDa), lysozyme (14 kDa) and coumaric acid (164 Da) were employed to calibrate the column. Absorbance of the eluted fractions was monitored at 280 nm.

Infrared spectra. IR spectra of EIF were performed in a Perkin Elmer 1310 spectrophotometer. Samples were prepared in KBr.

Decolorization assays. Decolorization of kraft lignin and Remazol Brilliant Blue were determined following the procedures described by Eaton *et al.* [9] and Glenn and Gold [12], respectively. The wavelenghts used to calculate the absorbance ratios of the latter were 585 and 500 nm. In both cases, a Shimadzu UV-visible recording spectrophotometer UV-160 was used.

High performance liquid chromatography (HPLC). Reversed phase HPLC was performed on a Shimadzu LC-6A liquid chromatograph attached to a LC-injector SIL-1A. A UV-visible Shimadzu SPD-6A UV spectrophotometric detector was fixed at 280 nm. Chromatographic separation was achieved using a stationary phase of 10 μm C18 μBondapak from Du Pont Instruments packed in a 4.6 mm × 15 cm stainless steel column. The mobile phase was methanol/H_2O (20/80%) with a flow rate adjusted to either 0.7 or 1.0 ml/min. Recording was performed on a Shimadzu C-R3A chromatopac recorder.

In order to improve the resolution of the components of the ESF, samples were pre-fractioned on a SEP-PAK column (1.0 ml) packed with the stationary phase indicated above, according to the protocol described by Goycoolea *et al.* [14]. Four fractions were obtained by elution of the SEP-PAK column with 1.0 ml of 0, 10, 20 and 50% methanol in water. These were acidified to pH 1.0–2.0 with HCl, extracted with diethyl ether and dried. Samples were resuspended in 50 μl of water and injection volumes ranged between 1.0 to 5.0 μl. Samples from eluates and washes with 10 and 20% methanol were eluted at a flow rate of 0.7 ml/min, whereas samples of the 50% methanol wash were eluted at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

1. Effect of adding ESF of EIF of kraft lignin to cultures of S. viridosporus T7A growing on yeast extract.

Flasks containing 9.0 ml of minimal medium supplemented with varying amounts of either ESF or EIF of kraft lignin and 0.3% of yeast extract were inoculated with spores and incubated at 37°C for 4 days. After preliminary experiments, EIF was tested in the range of 1.0 to 5.0 mg/ml, whereas ESF was assayed between 0.5 and 2.5 mg/ml. Mycelia were then collected and weighted as described above.

The two fractions of purified kraft lignin have different effects on the growth of *S. viridosporus* T7A on yeast extract. Based on biomass formation, growth differences between cultures with and without EIF were not significant using 0.3% yeast ex-

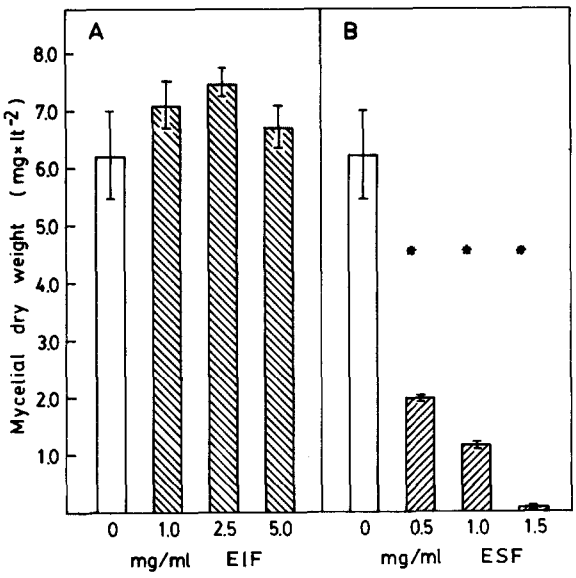

Fig. 1 Effect of purified fractions of kraft lignin on growth of *S. viridosporus* T7A. Bars represent dry mycelial weight recovered after 4 days of incubation on 0.3% of yeast extract. (A) Biomass determined after growth on culture medium supplemented with 0, 1.0, 2.5 and 5.0 mg/ml of EIF. (B) Biomass after growth on culture medium supplemented with 0, 0.5, 1.0 and 1.5 mg/ml of ESF. Values (triplicates) are expressed as the mean ± standard deviation and analyzed according to Student's *t* test. *: $p<0.005$ with respect to cultures lacking kraft lignin fractions.

tract (Fig. 1). With 0.1% yeast extract, growth differences in the presence of 1.0 and 5.0 mg/ml of EIF were slightly significant ($p>0.05$) when compared to cultures lacking EIF (data not shown). In contrast, ESF inhibits development of *S. viridosporus* T7A at either concentration of yeast extract. Fig. 1 shows bacterial biomass formation with 0.3% yeast extract plus increasing amounts of ESF. Addition of 0.5 mg/ml ESF inhibited growth to one third, with spores developing into small and scarce mycelial cells. Growth was totally supressed with 1.5 mg/ml ESF.

The reason for the different behaviour of *S. viridosporus* T7A with these two fractions of kraft lignin is not known. Due to the ability of this strain to metabolize small molecular weight lignin-derived compounds, we expected that ESF would provide a better source of growth substrates that EIF. It is possible that ESF may contain one or more inhibitors among its multiple components. However, these putative inhibitors would be selective for this strain, since other bacteria grow well on this fraction [13-14].

Gel permeation chromatography was employed to measure the molecular size distribution of EIF after incubation with *S. viridosporus* T7A. Cultures containing 0.3% of yeast extract supplemented with 2.5 mg/ml of EIF were incubated for 12 days. Full growth of *S. viridosporus* mycelia was achieved after approximately 48 h. Aliquots were withdrawn from the culture supernatants after 0, 4, 8 and 12 days of incubation and filtered through a Sephadex G-50 column as described above. No significant changes in the elution pattern of the substrate were observed with this methodology. Infrared spectra of the same samples were also essentially identical to those from uninoculated controls (data now shown).

2. HPLC of ESF before and after incubation with S. viridosporus T7A.

Previously, we have shown that HPLC constitutes a sensitive technique for analyzing bacterial consumption of ESF components [14]. Although ESF inhibits growth of *S. viridosporus* T7A, subtle changes might be produced in this fraction due to the action of this microorganism. To test this possibility, cultures containing 2.5 mg/ml ESF inoculated with spores and pre-grown mycelia were subjected to HPLC. For these analyses, ESF was prefractionated as indicated in Materials and Methods in order to improve resolution. Fig. 2 shows the elution patterns obtained for each of the four subfractions of both cultures, plus those derived from uninoculated controls. Both spores and mycelia were found to metabolize the peak of lower polarity of sub-fraction A. Also, both cultures caused the appearance of new peaks in sub-fraction B. Some quantitative changes are observed in the components of sub-fraction C, while subfraction D remains virtually unaffected.

The same procedure is not suitable for analyzing metabolism of EIF components [14]. However, it is conceivable that incubation of the bacterium in the presence of EIF could lead to the production of ether-soluble compounds detectable by HPLC anal-

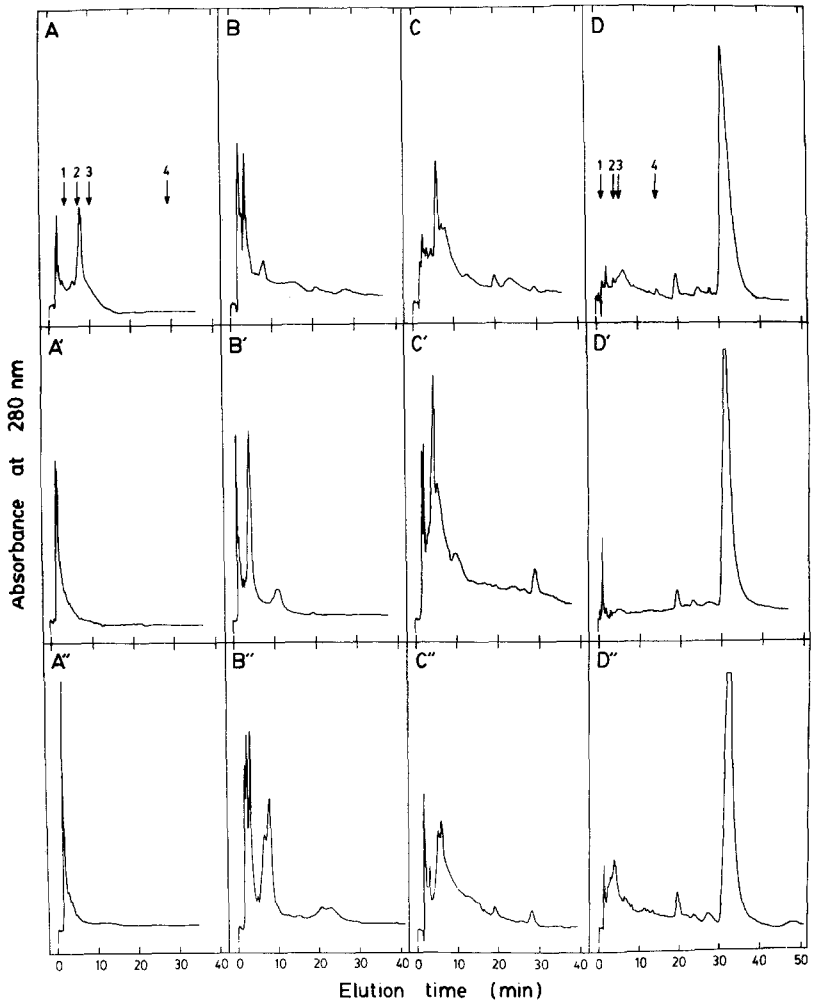

Fig. 2 HPLC elution profile of SEP-PAK precolumn fractions analyzed after growing *S. viridosporus* T7A on the ESF of kraft lignin. A–D: uninoculated controls; A′–D′: cultures inoculated with spores and A″–D″: cultures inoculated with mycelia. A,A′, A″: eluate; B,B′,B″: wash with 10% methanol. C,C′,C″: wash with 20% methanol; D,D′,D″: wash with 50% methanol. Elution times of standards are indicated by arrows: vanillate (1), coumarate (2), ferulate (3) and cinnamate (4).

ysis. *S. viridosporus* was therefore incubated in minimal medium containing 0.3% of yeast extract and 2.5 mg/ml of EIF at 37°C for 4 days. Aliquots of inoculated cultures and sterile controls obtained at the end of the incubation period were extracted with ether, evaporated and resuspended in water. One set of samples was acidified before extraction and another set of aliquots was extracted at neutral pH. In neither case were distinctive peaks visualized, indicating that degradation intermediates do not accumulate in the cultures.

3. Decolorization assays

As mentioned above, various fungal strains produce extensive bleaching of kraft pulp waste liquors [9–11, 18]. Based on this observation, the color of cultures of *S. viridosporus* T7A supplemented with 2.5 mg/ml of EIF was monitored during 21 days.

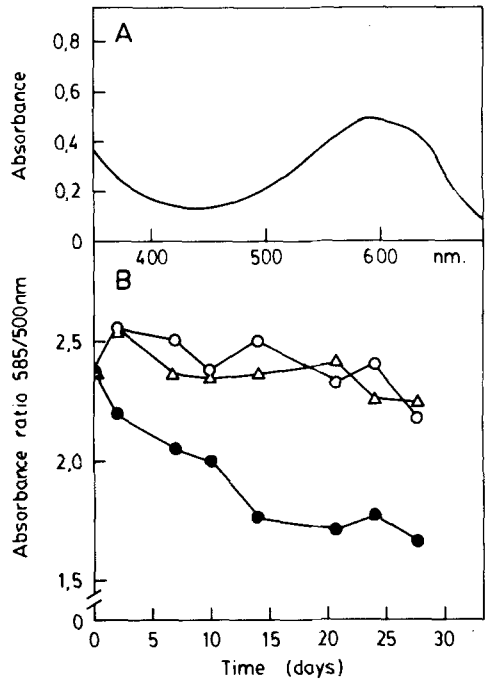

Fig. 3 Decolorization of Remazol Brilliant Blue R by *S. viridosporus* T7A. (A) Visible spectrum of Remazol Brilliant Blue R. (B) Decolorization expressed as the absorbance ratio at 585 and 500 nm of aliquots withdrawn from cultures with 0.05% of the dye. (○): uninoculated controls containing lignocellulose; (△): cultures of *S. viridosporus* T7A lacking lignocellulose; (●): cultures of *S. viridosporus* T7A with lignocellulose.

Incubations were carried out both in the presence and in the absence of lignocellulose, since this substrate induces some enzymatic activities of the streptomycete [22]. In both experiments the color of the media remained unaltered throughout the incubation period.

On the other hand, Glenn and Gold have presented evidence indicating correlation between fungal ligninolytic activity and decolorization of several polymeric dyes [12]. Thus, the use of these dyes should provide a simple and rapid assay for screening of ligninolytic microorganisms and quantification of their action.

We selected the dye Remazol Brilliant Blue R to investigate the decolorization ability of *S. viridosporus* T7A. In this case, partial decolorization occurred, although solely when the actinomycete was incubated in the presence of lignocellulose (Fig. 3). These results suggest that indeed lignocellulose induces the appearance of enzymes that are undetectable in its absence. At present their identity is unknown, although they might be related to the peroxidases mentioned above [22]. If this was the case, *S. viridosporus* T7A peroxidases would differ from their fungal counterparts in that the former are not able to act on kraft lignin.

Results reported in this work indicate that kraft lignin is not efficiently metabolized by *S. viridosporus* T7A. In addition, its failure to decolorize EIF indicates that this microoorganism is not suitable for treatment of waste liquors originated from pulp mills.

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