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Lignosulfonate Biodegradation by *Chrysonilia sitophila*

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ABSTRACT

The lignosulfonate biodegradation by *Chrysonilia sitophila* at three different nitrogen concentrations with and without oxygenation was studied. Significant transformation was observed at high nitrogen concentrations (76 mmol N/L) in the absence of oxygen flushing as observed by changes in the absorbance, fluorescence, and mol wt distribution. Phenoloxidase production was 1.8 U/L (*o*-dianisidine oxidation), and the maximum H_2O_2 (necessary to the action of peroxidase) production was obtained at 12th d in high nitrogen, oxygenated cultures. The pattern of the LS depolymerization was different with and without oxygenation. IR analysis of biodegraded LS showed a relative increase of sulfonate groups, compared with aromatic groups. A decrease of lignosulfonate was evident by the nitroso method.

Index Entries: Lignosulfonate biodegradation; *Chrysonilia sitophila;* phenol oxidase; lignin biodegradation; ascomycete.

INTRODUCTION

Chrysonilia sitophila (Mont von Arx) is a ligninolytic fungus (*1*), isolated from macerate of the insect *Tribolium ferrugineum,* found in rice hull coming from the state of Goiás, Brazil (*2*). The teleomorph of this species is *Neurospora sitophila* (*3,4*), a well-known ascomycete.

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At least three enzymes with ligninase activity are produced by this fungus (5). Cellulase activity and single cell protein production by *C. sitophila* have been reported (6). The optimal conditions for ligninase production or lignin degradation have not been established.

Studies with *Phanerochaete chrysosporium* demonstrated that there is parallelism between the physiological conditions for lignin degradation and those for lignosulfonate (7). Lignosulfonates (LS) are byproducts of the production of cellulose from wood and exhibit a higher recalcitrance toward biological attack than lignin *per se* (7–9).

The LS biodegradation by *Trametes versicolor* (10), *P. chrysosporium* (7,8,11), *P. chrysosporium* immobilized on foam (12), and *Trichoderma harzianum* (9) has been studied. Nitrogen concentration, agitation, and oxygenation are important parameters in the lignin biodegradation by *P. chrysosporium* and other white rot fungi (13). The influence of these factors on LS biodegradation by *C. sitophila* are discussed.

MATERIALS AND METHODS

Lignosulfonate (LS)

A commercial sodium LS, VIXIL S PC, of MELBAR Prod. de Ligninas Ltda., Brazil, was used.

Organism

C. sitophila TFB 27441 was isolated as described previously (2) and maintained in Fries agar plates at 5°C.

Media and Culture Conditions

Cultures were grown in unbuffered modified Fries medium (14) in 2-L Erlemeyer flasks at 28°C. Variable nitrogen concentrations as ammonium tartrate were added to this medium. Oxygen, when required, was flushed for 10 min on each of 3 d. LS at 0.1% and glucose at 1.0% were used. The cultures were agitated at 150 rpm on a rotary shaker.

Enzyme Assay

Phenol-oxidase from culture supernatants was measured according to the modified method of Eriksson et al. (15). To 1.5 mL of 1 m*M* *o*-dianisidine solution, 0.5 mL of H_2O_2 (1 m*M*) and 1 mL of broth culture were added. After 10 min at room temperature, the absorbance at 460 nm was measured (oxidized *o*-dianisidine absorbes at 460 nm with E = 29,460). One unit (U) was defined as 1 μmol of oxidized *o*-dianisidine in 1 min and the activities were reported as U/L. For laccase activity, H_2O_2 was not added.

Assay in plates for tyrosinase activity were prepared according to Ander and Eriksson's (*16*) methodology. Alcohol solutions of D-tyrosine and *p*-cresol were overlaid onto agar plates with actively growing mycelium.

Hydrogen Peroxide Determination (*17*)

H_2O_2 was quantified via the peroxidase-dependent oxidation of *o*-dianisidine. To 10 mL of culture filtrate, 0.5 mL of aqueous solution containing 3.1 μmol of *o*-dianisidine and 0.3 mg of peroxidase (type I, SIGMA) was added. Cultures were flushed with 100% oxygen for 15 min and then incubated at 39°C for 3 h. The absorbance at 460 nm against a water blank was measured.

Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) was performed using Sephadex G-50 gel in 53×1.5-cm column with aqueous solution of 0.03*M* NaCl, at pH 11 as eluent (*18*). The standards used were trypsin (MW 23,000), myoglobin (MW 17,000), Lysozyme (MW 13,900), Phlobaphene (MW 6,500). A LKB column, pump, and UV detector at 275 nm were used.

Lignosulfonate Determination

LS was determined by the colorimetric nitroso reaction followed by measurement at 440 nm (*19*). The LS adsorbed on mycelium was released as described by Kern (*9*). The UV and fluorescence spectra were carried out on an Intralab DMS-100 spectrophotometer and a Perkin Elmer MPF-44B spectrofluorimeter, respectively.

IR Analysis (*9*)

Filtrates from 12-d cultures were shaken with $CHCl_3$, dialysed against distilled water, and lyophilized. IR spectrum were recorded in KBr using a Perkin Elmer 1430 Spectrophotometer.

RESULTS AND DISCUSSION

In general, in the LS biodegradation, several simultaneous reactions happen: oxidation, depolimerization, and even, the complete mineralization to $CO_2 + H_2O$. Therefore, measures of the total LS biodegradation are difficult because the LS is changing. In this work, several different measures are made in order to show these changes. Direct evidence would be obtained using ^{14}C-labeled LS (*20*), but it was not available.

Figure 1 shows absorbance, fluorescence, and nitrosolignin changes and phenol-oxidase production in cultures incubated with LS at 76 mmol

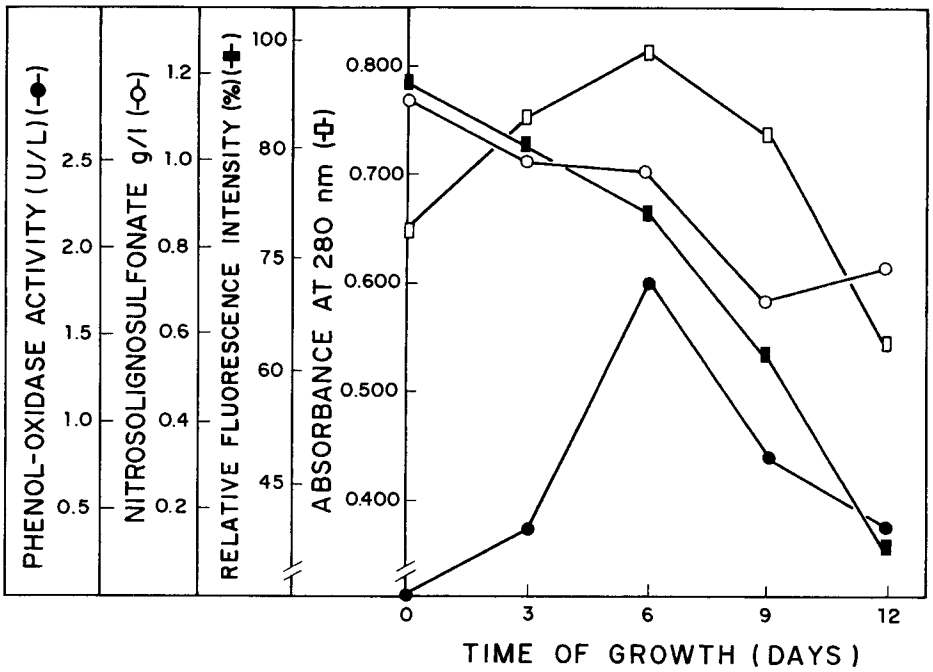

Fig. 1. Absorbance, fluorescence, nitrosolignosulfonate, and phenoloxidase production of *C. sitophila* cultures containing 0.1% of lignosulfonate and 76 mmol N/L, without oxygen flushing. Samples were diluted 10-fold from the original broth for measures of absorbance and fluorescence.

N/L without oxygenation. These parameters were measured in cultures at three different nitrogen concentrations (76, 27, and 9 mmol N/L) with and without oxygenation. The most pronounced changes were observed at 76 mmol N/L, and when the cultures were not oxygenated. No difference was observed in absorbance and fluorescence between stationary and agitated cultures (results not shown), therefore, just stationary cultures were used.

Although a high increase (about 30%) of the absorbance, at 76 mmol N/L was observed on the 9th d, under oxygenated conditions (not shown results), this did not decrease to levels observed when oxygen was not flushed.

The absorbance pattern of LS degradation was very similar to that described by Ulmer et al. (*11*), in their study of LS degradation by *P. chrysosporium*. This pattern could be attributed to the initial increase of chromophoric groups and posterior LS degradation.

A LS loss of 48% was measured using the nitroso reaction (Fig. 1), which is based on the reaction of nitroso acid with phenolic groups of LS and is used in the study of LS biodegradation (*9*). Less than 4% of mycelium adsorbed LS was detected by this method.

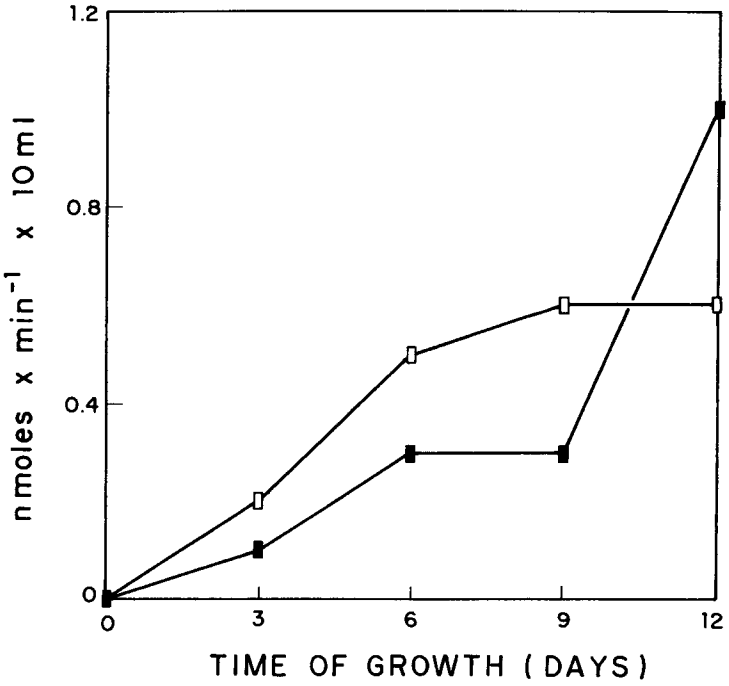

Fig. 2. Production of H_2O_2 by *C. sitophila* in the presence of 0.1% of LS and 76 mmol N/L with (—■—) and without (—□—) oxygen flushing.

The fluorescence spectrum of LS has a maximum at 400 nm when excited at 335 nm. The changes in the intensity of this spectrum at 400 nm during the biodegradation process are showed in Fig. 1. A decrease of 70% in fluorescence was observed when the nitrogen content was 76 mmol N/L. The changes were less pronounced when lower nitrogen concentrations were used and oxygen was flushed.

Phenol-oxidase enzyme activity, as shown in Fig. 1, has a maximum of 1.8 U/L on the 6th d. In the absence of H_2O_2, no activity was found. Plate assays showed no activity of tyrosinase enzymes using the specific reagents tyrosine and *p*-cresol.

Phenol-oxidizing enzymes have been shown to be important for lignin degradation (*16*). The detected phenol oxidase activity with the absence of laccase and tyrosinase activities indicated that only peroxidases were present. Owing to the strong absorbance at 310 nm, it was impossible to measure ligninase in the presence of LS.

The production of H_2O_2 at 76 mmol N/L increases with culture age, as shown in Fig. 2. Lower values were obtained at lower nitrogen concentration (results not shown), and just a small increase after the 9th d was observed when oxygen was flushed. This was different from the observation that H_2O_2 production by *P. chrysosporium* is dramatically increased with

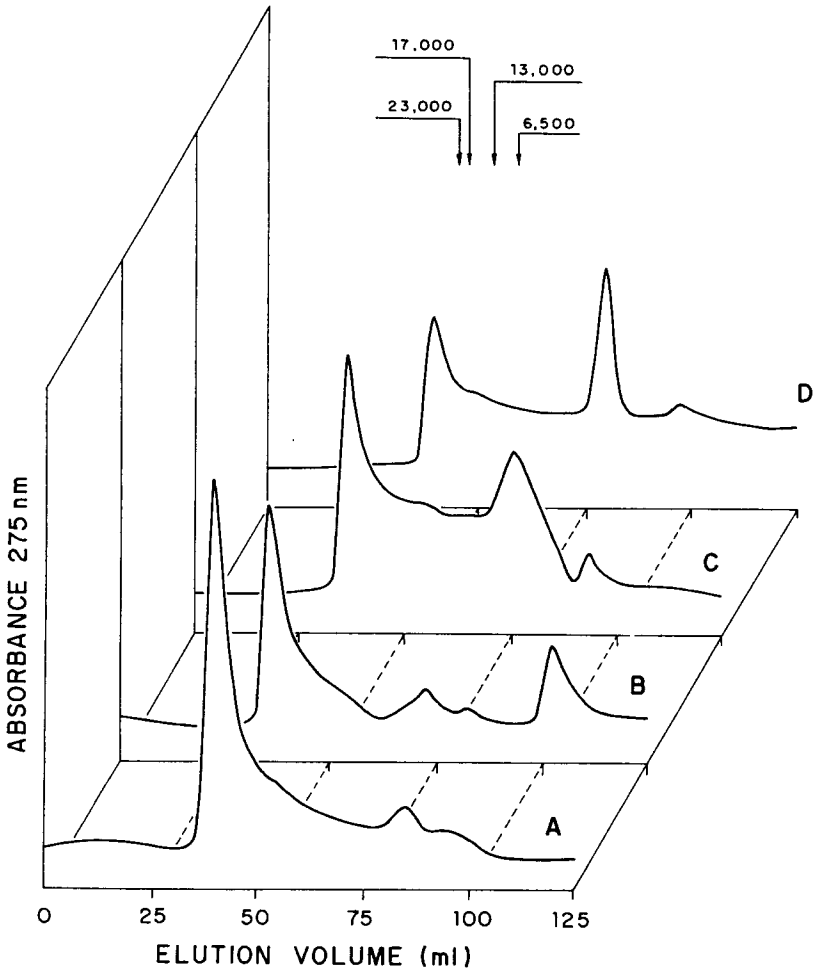

Fig. 3. Gel permeation chromatogram on Sephadex G-50 of *C. sitophila* cultures containing LS at different nitrogen concentrations without oxygen flushing. A: Noninoculated. B, C, and D: Cultures containing 9, 27, and 76 mmol N/L, respectively.

oxygen flushing (*17*). It is known that the H_2O_2 is necessary to peroxidase activity, which is unequivocally involved in the lignin biodegradation (*13*).

The gel permeation chromatogram of biodegraded LS shows that *C. sitophila* depolymerizes LS and that this depolymerization was maximal when nitrogen nutrient was in excess. A different profile for depolymerization can be observed with and without oxygenation (Figs. 3 and 4).

In order to describe structural changes in LS biodegradation, IR spectroscopy was used (*9*). Using the absorbance ratio at 1040 cm^{-1} (sulfonate)/ 1610 cm^{-1} (aromatic), a relative increase of sulfonate/aromatic groups from 0.34 to 0.48 in the untreated and biodegraded LS (76 mmol N/L, not oxygenated cultures), respectively, was observed.

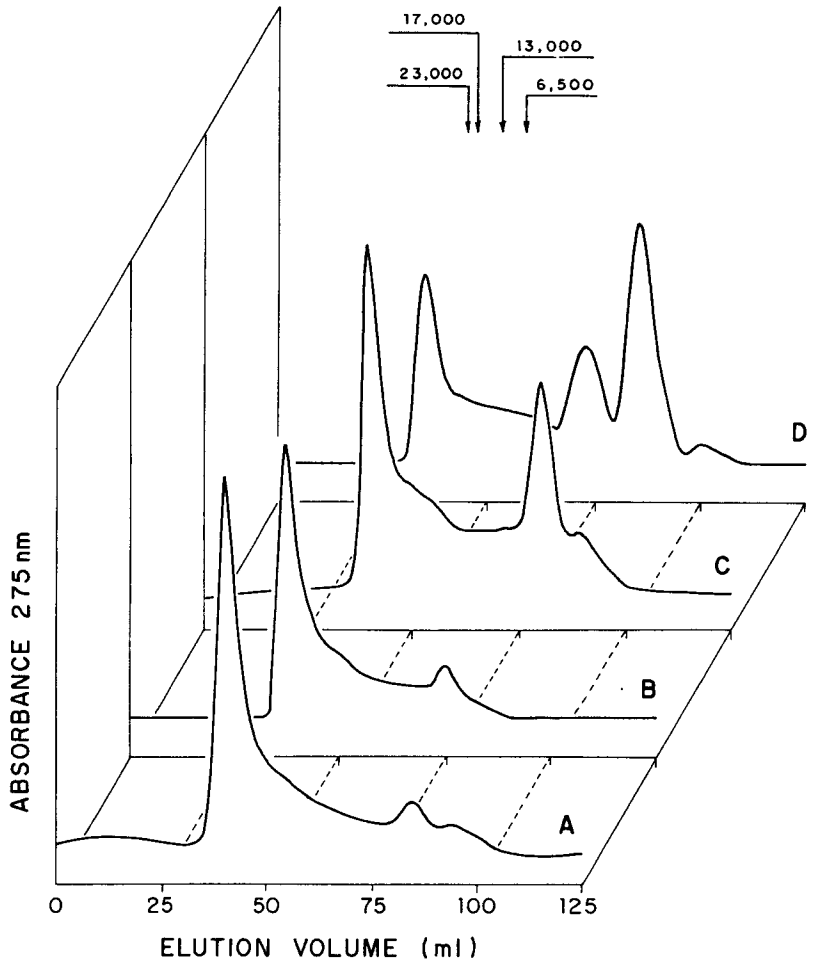

Fig. 4. Gel permeation chromatogram on Sephadex G-50 of *C. sitophila* cultures containing LS at different nitrogen concentration with oxygen flushing. A: Noninoculated. B, C, and D: Cultures containing 9, 27, and 76 mmol N/L, respectively.

The results reported here show that the LS biodegradation by the ascomycete *C. sitophila* is stimulated rather than inhibited by increased nitrogen nutrient. On the contrary, lignin degradation by *P. chrysosporium* and several other species of white-rot fungi are stimulated by *N*-limitation (*13*). Another exception as *C. sitophila* is *Lentinus edodes* (*21*), which expressed ligninase activity during the vegetative growth period.

The negative influence of oxygen on LS biodegradation by *C. sitophila* and on its phenol-oxidizing enzymes is not fully clear, and is still open to questions. However, inhibition by oxygen of ligninolytic enzymes production was also observed in *Coriolus versicolor* cultures (*22*).

In our knowledge, none of the ascomycetes, except *C. sitophila*, has been reported to produce ligninase, but phenoloxidases, i.e., *Podospora*

anserina, Neurospora crassa, Chaetomium piluliferum, Chaetomium cellulolyticum (23–25). Experiments in order to determine *C. sitophila* enzymes responsible by LS biodegradation are in progress in our laboratory, nevertheless, these results show that *C. sitophila* has good potential for applications in lignosulfonate biodegradation.

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