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Degradation of β-O-4 Lignin Model and Related Compounds by the Ascomycete *Chrysonilia sitophila* (TFB 27441 Strain)

RAQUEL F. P. NOGUEIRA,[1] RONALDO A. PILLI,[2] AND NELSON DURÁN[1,*]

Instituto de Quimica, [1]Biological Chemistry Laboratory and [2]Organic Synthesis Laboratory, Universidade Estadual de Campinas, C.P.6154, Campinas, CEP 13081, SP, Brazil

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ABSTRACT

Growth of the ascomycete *Chrysonilia sitophila* during degradation of lignin model dimers and monomers was compared to a glucose control. An inhibition of growth by Cα-carbonyl monomers and stimulation by β-O-4 lignin model and vanillyl alcohol were observed. A comparison of the degradation by this ascomycete with the basidiomycete *Phanerochaete chrysosoporium* showed similarities in relation to the type of degradation caused.

Index Entries: Lignin degradation; β-O-4 lignin model; ascomycete; *Chrysonilia sitophila.*

INTRODUCTION

Recent investigations employing lignin model compounds are elucidating the specific reactions of lignin degradation by ligninolytic microorganisms (*1,2*). *Chrysonilia sitophila* (TFB 27441) is an ascomycete, isolated from a xylophagous insect (*3,4*), whose telemorphic state is *Neurospora sitophila,* a well known fungus that has been used in the production of

*Author to whom all correspondence and reprint requests should be addressed.

Indonesian "ontjohn" for human food (*5*). *Chrysonilia sitophila* has demonstrated high ligninolytic activity from which three hemoproteins with ligninase properties were purified by ion-exchange chromatography and good quality single-cell proteins were produced (*6,7*). In this work, we have studied the degradation of different lignin model compounds by intact cells of the *C. sitophila*.

MATERIALS AND METHODS

Substrates

The β-O-4 lignin models IV and V (Fig. 1) were synthesized as described previously (*8*). Vanillyl alcohol was synthesized by reduction of vanillin by *Saccharomyces cereviseae* by the procedure of De Wulf and Thonar (*9*). Veratraldehyde was prepared by methylation of vanillin (*10*). The other substrates were purchased from Aldrich Chem. Co. Inc.

Culture Conditions

Comparative Growth

The *C. sitophila* was inoculated into 20 mL of medium containing $(NH_4)SO_4$, 5.6 g/L; K_2HPO_4, 1.0 g/L; $MgSO_4$, 0.5 g/L; NaCl, 0.1 g/L; $CuSO_4 \cdot 5H_2O$, 0.1 mg/L; $FeSO_4$, 0.20 mg/L; $MnSO_4$, 0.20 mg/L; $ZnCl_2$, 0.15 mg/L; 0.1 or 0.3% of the substrates; and 0.1% (w/v) of glucose at pH 6.0. Triplicate cultures were incubated without agitation at 28°C for 5 d, and then were filtered, dried at 80°C overnight, and weighed to determine the mycelium dry wt. Glucose controls and uninoculated controls were treated similarly.

Degradation of β-O-4 Model Dimers

The *C. sitophila* was incubated for 3 d in 20 mL of the medium described above with 1% (w/v) of glucose in 0.025*M* phthalate buffer, pH 6.0. After this time, the medium was removed, and the mycelium was washed thoroughly with sterile water to remove the buffer completely. The substrates were added as suspension in *N,N*-dimethylformamide to 20 mL of medium without buffer (pH adjusted to 6 with 1.0*M* NaOH) to give a final concentration of 0.02%. Glucose, uninoculated controls, and test cultures were incubated for 4 or 7 d at 28°C.

Extraction and Identification of Metabolites

After incubation, the cultures were filtered, acidified with 6*N* HCl to pH 2, and then extracted three times with ethyl acetate. Combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The residue was dissolved in methanol for high performance liquid chromatography (HPLC) and UV absorption spectroscopy.

The HPLC analyses were performed with a liquid chromatograph (Waters 6000) equipped with an UV detector operating at 280 nm. The

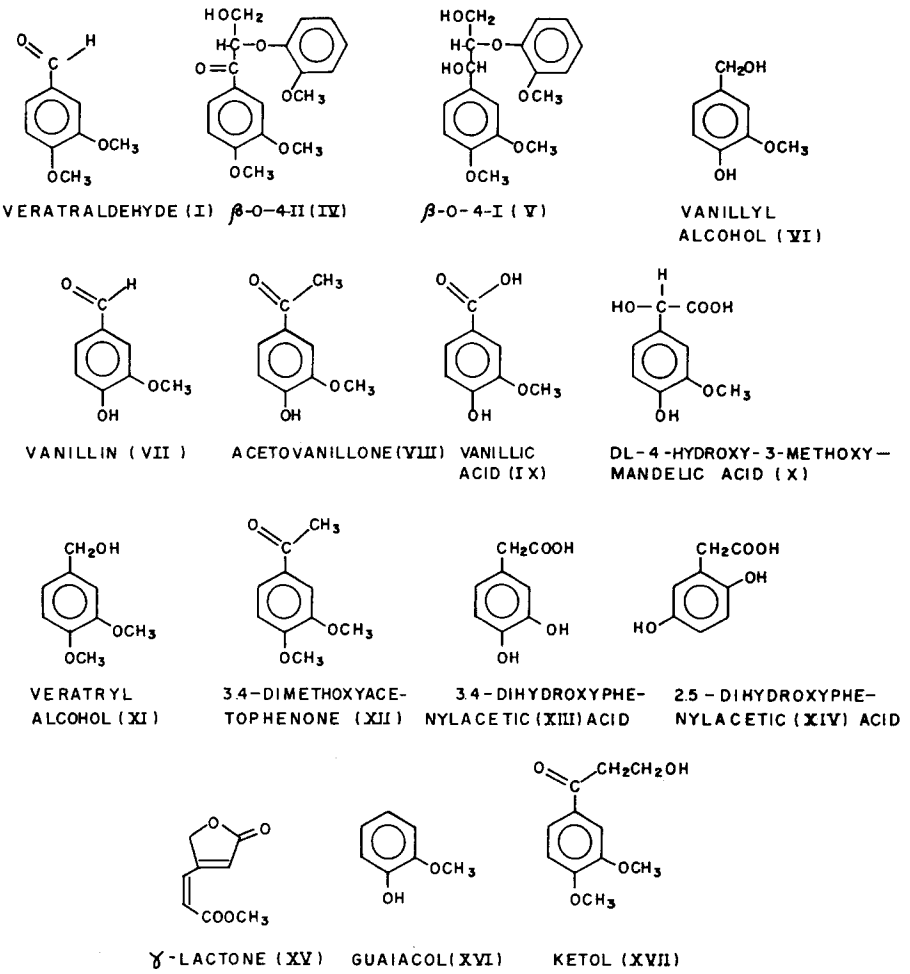

Fig. 1. Lignin model compounds used and some products of the biodegradation by *C. sitophila.*

mobile phase used was methanol:water (1:1). A 25 μL sample was injected onto a C_{18} Bondapack column; the flow rate was 0.5 or 1.0 mL/min. The chromatographic peaks were identified by comparison with standards when available (Fig. 1).

RESULTS AND DISCUSSION

Comparative Growth of *C. sitophila* on Different Lignin Compounds

Because our previous work (*4*) showed that the highest production rate of ligninase activity was observed after 5 d and that high rate lignin transformation occurred in the presence of *C. sitophila,* we have selected the same period for the degradation of the lignin models (*4,5*).

Table 1
Mycelium Dry Wt of *C. Sitophila* After 5 d of Incubation with Different Lignin Model Compounds (0.1% w/v)

Compound	Mycelium dry wt, mg	Substrate degradation, %[b]
Control	5.06±0.68[a]	ND
Veratraldehyde (I)	2.39±1.64	ND
β-O-4-I (V)	7.56±1.70	84
Vanillin (VII)	1.67±1.21	ND
Acetovanillone (VIII)	2.17±0.96	ND
Vanillic acid (IX)	1.32±0.07	ND
DL-4-Hydroxy-3-Methoxymandelic acid (X)	4.03±0.09	ND
Veratryl Alcohol (XI)	5.54±0.22	68
3,4-Dimethoxyacetophenone (XII)	4.08±1.17	ND
3,4-Dihydroxypenylacetic acid (XIII)	7.36±1.35	86
2,5-Dihydroxypenylacetic acid (XVI)	5.92±0.28	ND

[a] Average values of triplicated determinations.
[b] Followed by HLPC.
ND not determined.

Table 1 presents the results for the growth of *C. sitophila* determined by the mycelium dry wt in this period. The best substrates, at the concentration of 0.1% (w/v), were 3,4-dihydroxyphenylacetic acid (XIII), veratryl alcohol (XI) 2,5-dihydroxyphenylacetic acid (XIV), and β-O-4-I (V). These induced growth superior to that of the glucose control, indicating almost complete degradation of these compounds by the fungus, as demonstrated by HLPC analyses.

When the concentration was increased to 0.3%, the growth was completely inhibited by veratraldehyde (I), vanillin (VII), acetovanillone (VIII), vanillic acid (IX), and 3,4-dimethoxyacetophenone (XII) (results not shown).

According to Kirk and Farrel (*11*), whether an aromatic ring can be a substrate for ligninase, depends in part on the oxidation potential. Strong electron-withdrawing substituents, such as Cα-carbonyl groups, inactivate aromatic nuclei to ligninase, whereas alkoxyl groups activate them. In our case, the compounds that inhibited fungal growth are those that have a C-α-carbonyl group, suggesting that this group exerts a toxic effect on *C. sitophila*.

Degradation of Lignin Models

The UV absorbance at 280 nm and HPLC analysis indicated metabolism of compounds (XIII) and (XI) when initial concentration was 0.1% (w/v). In the case of compound XIII, no product was detected by HPLC, probably indicating a complete mineralization ($CO_2 + H_2O$) or intracellular

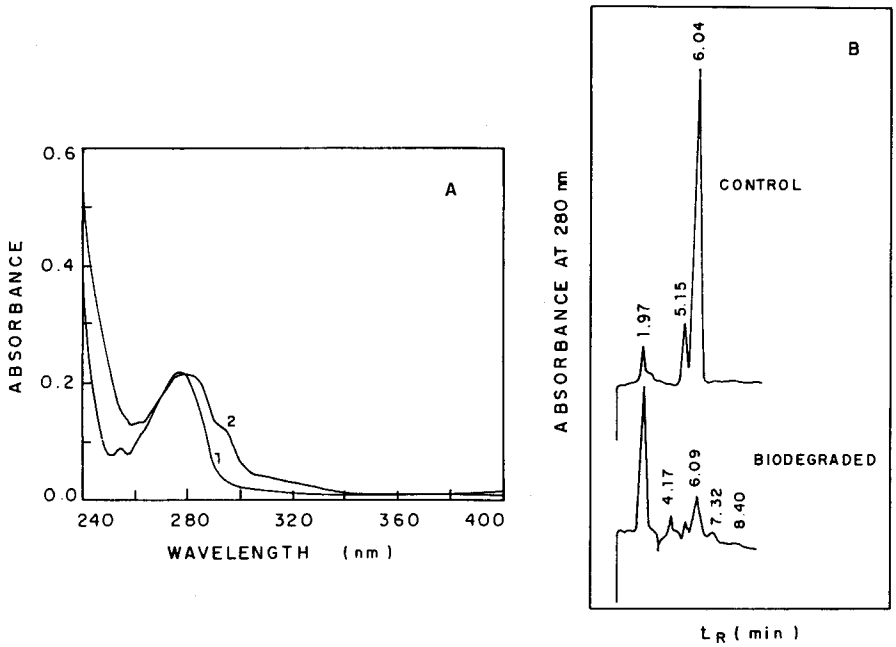

Fig. 2. **A:** UV absorption spectra of compound β-O-4-I (V), control (1) and after 4 d of degradation (2) by *C. sitophila*. **B:** HPLC of compound V, control and degraded; flow rate, 0.5 mL/min.

catabolism (results not shown). This is not the case for compound XI, where besides the decrease in concentration, two products were detected (results not shown). One of them is the lactone XV (retention time 7.4 min) originating from aromatic cleavage as previously proposed for *P. chrysosporium* (*12*).

In the degradation of veratryl alcohol by *C. sitophila*, the formation of veratraldehyde was not observed as it is for *P. chrysosporium*. However, when the alcohol was added to a 5-d-old *C. sitophila* culture, a rapid oxidation of the alcohol to the aldehyde was observed. This suggests that it is being catabolized intracellularly by an oxidoreductase that reduces it rapidly back to the alcohol that accumulates in the culture.

We suggest that the major pathway for veratryl alcohol utilization by *C. sitophila* is the transformation of veratryl alcohol to the lactone and that the minor one is the transformation to the aldehyde, which probably is rapidly reduced back to the alcohol.

After degradation of the dimer β-O-4-I (V), a shoulder appeared in the UV spectrum around 290 nm (Fig. 2A). An HPLC analysis showed the formation of veratryl alcohol (4.17 min), guaiacol (4.7 min), a compound with retention time 7.32 min, and the C oxidation (β-O-4-II) product with retention time 8.4 min (Fig. 2B). The compound with retention time 7.32 was identified as derived from the degradation product β-O-4-II (IV).

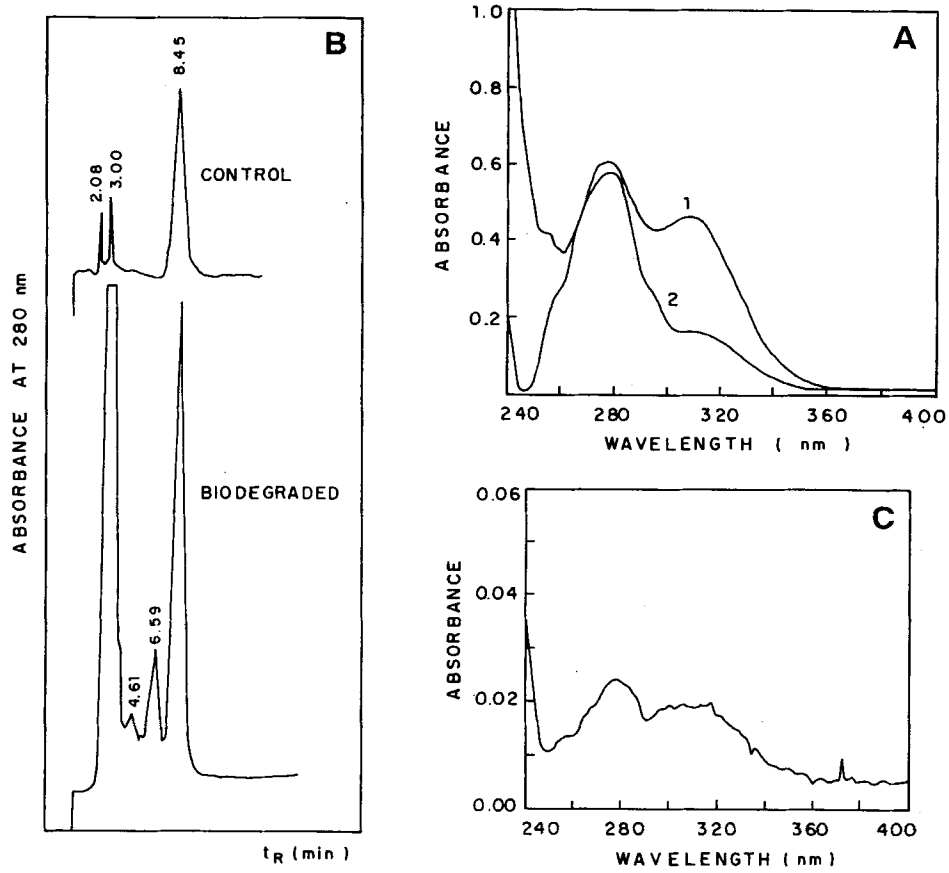

Fig. 3. **A:** UV absorption spectra of compound β-O-4-II (IV), control (1) and after 7 d of degradation (2) by *C. sitophila*. **B:** HPLC of compound IV, control and biodegraded; flow rate, 1.0 mL/min. **C:** UV absorption spectrum of compound with retention time 6.59 min.

In the degradation of β-O-4-II (IV) by *C. sitophila,* the UV spectrum after biodegradation showed a considerable decrease in the absorption at 310 nm that tends to disappear (Fig. 3A). An HPLC analysis showed the formation of two products, guaiacol at 4.61 min and another at 6.59 min (Fig. 3B). The compound with retention time 6.59 min (equivalent to 7.3 in Fig. 2B because of different flow rate) was isolated by preparative HLPC, and its UV spectrum (FIg. 3C) showed absorption maxima at 275 and 303 nm with a ratio $A_{275}/A_{303} = 1.27$. A similar UV spectra was found for 3,4-dimethoxyacetophenone (XII), which has absorption maxima at 274 and 303 nm with a ratio $A_{274}/A_{303} = 1.38$.

In order to corroborate this product distribution, we have carried out the degradation of β-O-4-I (V) by *P. chrysosporium,* since the products are known (*13,14*). The UV spectrum shows that degradation of compound V by *P. chrysosporium* and *C. sitophila* is identical (results not shown). An HLPC analysis shows the appearance of the Cα-oxidation product at 8.4

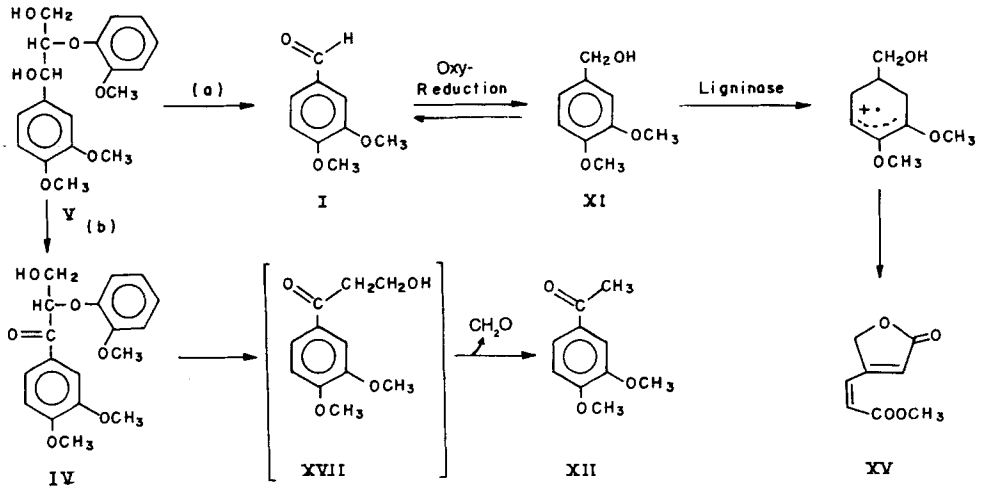

Fig. 4. Hypothetical degradation of β-O-4 compound by *C. sitophila*.

min (β-O-4-II), guaiacol at 4.7 min and veratryl alcohol at 3.7 min (results not shown). Therefore, we suggest that the degradation of β-O-4-II (IV) takes place in two steps, 3,4-dimethoxyacetophenone (XII) being the final product that originates from the ketol (XVII) and loss of formaldehyde, which also corresponds to the direct degradation product of β-O-4-I (V) with retention time 7.32 min. Additionally, it explains the low concentration of Cα-oxidation product in the degradation of β-O-4-I (V) owing to the rapid degradation to 3,4-dimethoxyacetophenone (XII) (with retention time 7.32 min) and guaiacol.

A general view for lignin model compounds degradation by *C. sitophila* is presented in Fig. 4. Since the main product from degradation of veratryl alcohol (XI) was the lactone (XV) and only veratryl aldehyde was detected in old cultures (there was a rapid reduction to the alcohol in actively metabolizing cell culture), the pathway (A) was important. The other important process in the β-O-4 metabolism was pathway (B). β-O-4-I (V) was rapidly transformed to the oxidized form [β-O-4-II (IV)], which immediately was transformed through to a hypothetical ketol (XVII) intermediate to the 3,4-dimethoxyacetophenone (XII). Then we conclude that the degradation of dimers β-O-4-I and II by an ascomycete was similar to a basidiomycete, and this is probably the mechanism of lignin degradation as observed recently on softwood chlorolignins biodegradation by *C. sitophila* (*15,16*).

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