0273–2289/93/4301—0037$03.00

Phenol Oxidases Production and Wood Degradation by a Thermophilic Fungus *Thermoascus aurantiacus*

ANGELA MACHUCA AND NELSON DURÁN*

Instituto de Quimica, Biological Chemistry Laboratory, Universidade Estadual de Campinas, CP 6154, Campinas, CEP 13081-970, S.P., Brazil

Received October 26, 1992; Accepted April 9, 1993

ABSTRACT

The ability of a Brazilian strain of *Thermoascus aurantiacus*, a thermophilic fungus, to produce extracellular phenol oxidases and to degrade *Eucalyptus grandis* sawdust was studied. *T. aurantiacus* was capable of good growth in liquid culture containing 1.5% (w/v) of various lignocellulosic substrates (sugar cane bagasse, rice hulls, and chips and sawdust of *E. grandis*) plus 5 mg/mL of glucose. When lignocellulosic substrates were used, enzymes involved in cellulose and hemicellulose metabolism were stimulated in *T. aurantiacus*. It was also found that these substrates have an inductive effect on phenol oxidase production. The most effective inducer of phenol oxidase activity was *E. grandis* sawdust, which led to the production of 0.80 U/mL (*o*-dianisidine oxidation) on day 12. Low phenol oxidase activity was observed at cultures when only glucose was used. Cultures of *T. aurantiacus* also exhibited cellobiose-quinone oxidoreductase activity when lignocellulosic materials were used as substrate. However, under our experimental conditions, lignin peroxidase activity was not detected. *E. grandis* sawdust supplemented with 5 mg/mL of glucose suffered a total weight loss of 6.7% accompanied by 15% lignin loss and 64.4% extractive loss after 21 d incubation with *T. aurantiacus*.

Index Entries: *Thermoascus aurantiacus;* phenol oxidases; thermophilic fungus; *Eucalyptus grandis* decay; cellulases; xylanases.

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

INTRODUCTION

Thermophilic fungus in association with other microorganisms are responsible for spoilage of bagasse, grain, hay, other agricultural residues, and industrial wood chips (*1*). Because thermophilic fungi are capable of producing thermostable enzymes suitable for use in processes of hydrolysis at elevated temperatures, these fungi are of particular interest in an industrial environment (*1*).

There are many reports of the ability of *Thermoascus aurantiacus*, a thermophilic ascomycete, to produce cellulases, xylanase, and amylase (*2–7*). However, the production of the extracellular phenol oxidases is not recorded, and little attention has been given to the role of this fungus in wood degradation. Thus, there is a need to study the degradation of wood and lignin, and the enzymes that take part in these processes in *T. aurantiacus*. The phenol oxidases (laccases and peroxidases) are essential in lignin degradation (*8–12*). However, these enzymes are probably not the only ones involved in lignin degradation by white-rot fungi. Other enzymes are also necessary to attack the various toxic quinones and phenoxy radicals that spontaneously polymerize and are formed from lignin by the action of phenol oxidases, e.g., cellobiose-quinone oxidoreductase (CBQase) and NAD(P)H quinone oxidoreductase (*13,14*). The interactions among laccase, different peroxidases (manganese-dependent peroxidase, lignin peroxidase, and horseradish peroxidase), and cellobiose-quinone oxidoreductase were recently reported by Ander et al. (*15*) and Samejima and Eriksson (*16*).

Previous studies in paper-making using Eucalyptus wood and a Brazilian strain of *T. aurantiacus* showed an improvement in cellulose yield and a reduced consumption of chemicals in the pulping processes (*17,18*). In order to clarify the mechanism involved, the current study focused on characterizing the cellulase, xylanase, and phenol oxidase activities produced by *T. aurantiacus* in liquid stationary culture in the presence of insoluble lignocellulosic substrates. This communication is the first report on phenol oxidase activity produced by a thermophilic fungus, *T. aurantiacus*, showing an enzymatic profile different from that reported in the other strain of this fungus (*2–7*). Since the main components of the lignocellulosic materials degraded by *T. aurantiacus* are compounds of relatively high molecular weight related to the extractive part (biflavonoids, hydrolyzable tannins, etc.) (*19*), the intracellular enzymes were not studied. In addition, *Eucalyptus grandis* sawdust was exposed to *T. aurantiacus* to study the wood-degrading ability of the fungus and its selectivity in order to evaluate its potential use in kraft pulping as a biological pretreatment.

MATERIALS AND METHODS

Microorganism

Thermoascus aurantiacus, a Brazilian strain isolated from Eucalyptus chips piles stored outdoors, was obtained from C. G. Auer of ESALQ, Piracicaba, S.P., Brazil (*20*). The strain was maintained on Czapek agar plates (*21*) at 4°C.

Culture Conditions for Enzyme Production

The fungus was cultivated in modified Czapek broth in Erlenmeyers flask (100/250 mL). The culture medium contained (g/L): glucose, 5.0; $NaNO_3$, 2.0; $MgSO_4$, 0.5; K_2HPO_4, 1.0; KCl, 0.5; $CaCl_2$, and 0.3 and 0.1 mL of trace elements according to DIFCO (*21*). The medium was adjusted to pH 6.0 before autoclaving at 121°C for 20 min. The inoculum was prepared by growing the fungus on Czapek agar plates at 48°C for 3–4 d, after which agar disks (6 mm diameter) were transferred to Czapek broth medium (1 disk/100 mL medium) for enzyme production.

Enzyme Production on Different Substrates

The enzyme production was studied in Czapek medium containing glucose 5 mg/mL, pH 6.0, and 1.5% (w/v) of the following lignocellulosic substrates: chips and sawdust of *Eucalyptus grandis,* and rice hulls or sugar cane bagasse. Samples of the culture media were taken periodically and filtered through Millipore membranes (pore size, 0.45 μm). Triplicate flasks were analyzed for each substrate. Fungal biomass was determined in terms of dry weight after mycelium removal from lignocellulosic substrate by a physical separation.

Enzyme Assays

Extracellular enzymes activities were measured directly using the filtered culture broth. Phenol oxidase (oxidase-like) was assayed by measuring the oxidation of 1.0 m*M* of *o*-dianisidine ($\epsilon_{460} = 29{,}400 M^{-1} \times cm^{-1}$) or syringaldazine ($\epsilon_{525} = 65{,}000 M^{-1} \times cm^{-1}$) according to Szklarz et al. (*22*) at pH 3.0. The activity of peroxidase was similarly measured, except that the mixture also contained 2.0 m*M* H_2O_2. Ligninase activity was determined by the veratryl alcohol oxidation method (*23*). Cellobiose quinone oxidoreductase (CBQase) activity was determined by measuring reduction of *p*-benzoquinone at 290 nm (*13,* modified method). Cellulolytic (CMCase) and xylanolytic (Xyl) activities were assayed according to Mandels et al.

Table 1
Effects of Different Substrates on Growth and Production of Cellulase and Xylanase (U/mL) by *Thermoascus aurantiacus*[a]

Substrate, 1.5%	CMCase	Xyl	β-gluc	Dry wt, mg
Rice hulls	0.58	0.20	0.040	34.7 ± 0.9
Bagasse	0.46	0.37	0.060	41.6 ± 0.7
Chips	0.10	0.15	0.001	35.7 ± 0.4
Sawdust	0.12	0.32	0.020	43.7 ± 1.0
Glucose[b]	0.07	0.05	0.000	42.0 ± 0.5

[a]Each value is the average of three replicates consistent within ± 5%. Enzymes activities (U/mL) were measured on culture filtrates of fungus grown 10 d on 1.5% of various lignocellulosic substrates supplemented with glucose (5 mg/mL).
[b]Culture containing only glucose (5 mg/mL or 0.5%) as substrate.

(*24*) and Khandke et al. (*5*), respectively. Reducing sugars released in the hydrolysis reaction were measured by the 3,5-dinitrosalicylic acid (DNS) method (*25*). Glucose and xylose were used as standards (550 nm). β-Glucosidase (β-glu) was determined according to Tan et al. (*3*) using *p*-nitrophenyl-β-D-glucopyranoside. One unit of all enzyme activities was expressed as micromoles of substrate reduced (CBQase activity) or oxidized (phenol oxidase activity), or product released (CMCase, Xyl and β-Glu activities)/min/mL of culture filtrate.

Degradation of Wood

Each 500-mL Erlenmeyer flask, containing 2.0 g of *E. grandis* sawdust (45–60 mesh) moistened with 40 mL Czapek solution (glucose 5 mg/mL, pH 6.0), was inoculated with three mycelial disks. Uninoculated sawdust served as the control. Triplicate flasks were processed after 21 d of incubation at 48°C as stationary cultures. Wood samples were dried to constant weight to determine the reduction in mass and used for chemical analysis. Klason lignin and extractive values were determined by the ASTM Standard Methods, D-1106-56 and D-1105-56, respectively. Cellulose value was determined as described by Pereira and Sardinha (*26*). Hemicellulose value was found by the difference with the total components.

RESULTS AND DISCUSSION

Good growth was obtained when both lignocellulosic materials and glucose were used as substrates. The cellulolytic and xylanolytic activities produced by *T. aurantiacus* in static cultivation were produced when the fungus was grown on 1.5% (w/v) lignocellulosic materials supplemented with 5 mg/mL glucose (Table 1). Enzyme activities were lower with only glucose as substrate. Culture filtrates of the fungus exhibited a low β-glucosidase activity.

Table 2
Effects of Different Substrates on Phenol Oxidase and CBQase Production (U/mL) by *Thermoascus aurantiacus*

Substrate, 1.5%	Oxidase activity *o*-Dian/Syrin[a]		Peroxidase activity *o*-Dian/Syrin		CBQase
Rice hull	0.610	0.014	0.210	0.008	0.009
Bagasse	0.630	0.018	0.130	0.007	0.010
Chips	0.079	0.000	0.000	0.000	n.d.[b]
Sawdust	0.710	0.075	0.000	0.000	0.016
Glucose	0.040	0.00	0.000	0.000	n.d.

[a]*o*-Dian = *o*-dianisidine and syrin = syringaldazine assays. The conditions are the same as in Table 1.
[b]Activity not determined.

Table 2 summarizes the results obtained for the phenol oxidases and cellobiose quinone oxidoreductase (CBQase) activities. Addition of the lignocellulosic substrates to cultures of *T. aurantiacus* was essential for the induction of phenol oxidases. In the medium containing glucose (5 mg/mL), these activities were much lower.

The type of phenol oxidase activity (oxidase or peroxidase) depends on the lignocellulosic substrate. Thus, oxidase and peroxidase activities were detected when rice hulls and sugar bagasse were employed, whereas with *E. grandis* wood, only oxidase was detected. The higher oxidase activity was obtained when *E. grandis* sawdust was used. The optimum pH for phenol oxidase was pH 3.0, with *o*-dianisidine as substrate.

CBQase was also detected when lignocellulosic substrates were used. It occurs in several fungi when grown on cellulose and/or lignin (*27–29*). Although not required for lignin degradation, it has been suggested to prevent polymerization of phenols and the accumulation of quinones formed by the action of phenol-oxidizing enzymes (*13,30,31*). This enzyme may therefore play an important role in the degradation of lignocellulosics by *T. aurantiacus*. Lignin peroxidase was absent throughout the entire culture period with all substrates, as determined by oxidation of veratryl alcohol into veratraldehyde.

When *E. grandis* sawdust (1.5%) supplemented with glucose was used as substrate, extracellular oxidase activity appeared early reaching the maximum on day 12 (0.80 U/mL, *o*-dianisidine oxidation), at which stage the reducing sugars reached a minimum level (0.19 mg/mL) (Fig. 1). Although the *o*-dianisidine oxidation assay gave higher values than the syringaldazine oxidation assay, identical activity profile curves were obtained with both substrates, indicating that these were perhaps oxidized by the same enzyme. Other substrates oxidized by culture filtrates stimulated with *E. grandis* sawdust included: guaiacol, catechol, orcinol, and Remazol brilliant blue. However, using DL-DOPA as substrate, no oxidation was observed. Phenol oxidase was completely inhibited by sodium

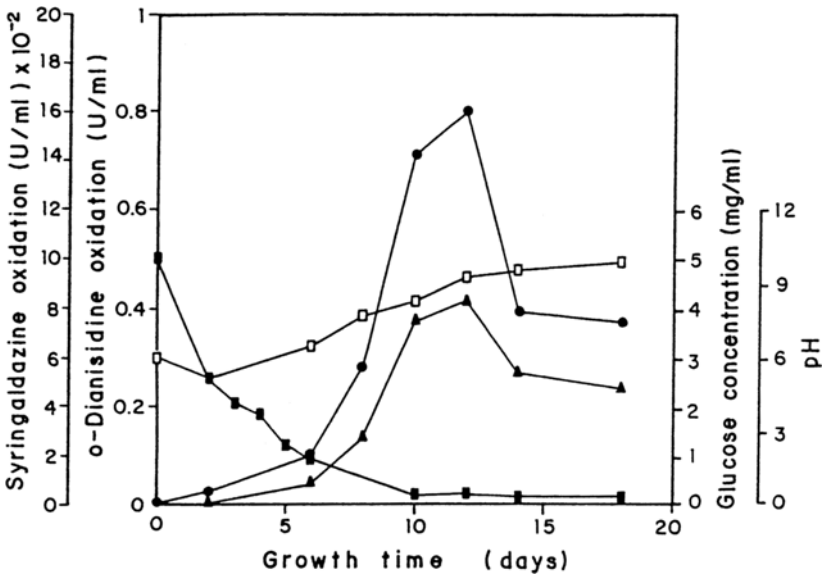

Fig. 1. Production of phenol oxidases (U/mL) by *Thermoascus aurantiacus* using *Eucalyptus grandis* sawdust (1.5%) supplemented with glucose (5.0 mg/mL) as substrate. Phenol oxidase activity determined by *o*-dianisidine (—●—) and syringaldazine (—▲—) oxidation. Change in the pH (—□—) and reducing sugars expressed as mg/mL of glucose (—■—) through the growth period (*see* Culture Conditions in the Materials and Methods section).

azide, which is a classical inhibitor of metal-containing oxidases (results not shown).

Addition of catalase (0.01 mg/mL, 250 U/mg) to the reaction had no effect on the phenol oxidase activity, excluding a role of H_2O_2 in the *o*-dianisidine and syringaldazine oxidations catalyzed by the *T. aurantiacus* enzymes. This is a strong indication that these enzymes are not dependent on H_2O_2 and are almost exclusively oxidase, possibly the laccase type. Further studies will focus on whether phenol oxidases are of laccase or peroxidase type.

Growth of *T. aurantiacus* on *E. grandis* sawdust for 21 d resulted in a 6.7% loss of the initial weight of the wood (Table 3). The level of cellulose showed no change after treatment with fungus. *T. aurantiacus* degraded only 15% lignin with respect to the control. The rapid decrease in extractives (ethanol/benzene/hot water) indicates that the fungus uses soluble material more efficiently than other wood components. The extractive loss was 64.4% in the 21-d period. This is very significant since the extractives have a damaging influence on kraft pulping, affecting yield, consumption of chemicals, and black liquor processing.

In conclusion, our results indicate that the lignocellulolytic activity of *T. aurantiacus* in liquid culture is stimulated by the presence of lignocellulosic substrates. Addition of *E. grandis* sawdust to the culture caused

Table 3
Percent Loss of *Eucalyptus grandis* Components Decayed by *Thermoascus aurantiacus* after 21 Days' Growth[a]

Percent loss	Decayed wood
% Weight	6.70 ± 0.7
% Extractives	64.4 ± 1.0
% Lignin	15.0 ± 1.1
% Cellulose	0.00
% Hemicellulose	11.0 ± 1.0

[a]Results are the average of three replicates ± SD related to sound wood.

increase of the phenol oxidase activity of approx 20-fold compared with the culture containing only glucose as substrate. The percent loss of 15% lignin and 64.4% extractives in wood decay after 21 d may be attributed to the high phenol oxidase activity produced by *T. aurantiacus*. Because of its properties, the Brazilian strain of *T. aurantiacus* appeared as a potential source in a pretreatment of wood in pulping processes and treatment of kraft mill effluents. Such studies are in progress.

ACKNOWLEDGMENTS

This work was supported by the CNPq, CAPES, PADCT, and FAPESP.

REFERENCES

1. Sharma, H. S. S. (1989), *Appl. Microbiol. Biotechnol.* **31,** 1.
2. Kawamori, M., Takayama, K., and Takasawa, S. (1987), *Agric. Biol. Chem.* **51,** 647.
3. Tan, L. U. L., Mayers, P., and Saddler, J. N. (1987), *Can. J. Microbiol.* **33,** 689.
4. Feldman, K. A., Lovett, J. S., and Tsao, G. T. (1988), *Enzyme Microbiol. Technol.* **10,** 262.
5. Khandke, K. M., Vithayathil, P. J., and Murthy, S. R. (1989), *Arch. Biochem. Biophys.* **274,** 491.
6. Adams, P. R. (1991), *Biotechnol. Appl. Biochem.* **13,** 430.
7. Adams, P. R. (1992), *Biotechnol. Appl. Biochem.* **15,** 311.
8. Ander, P. and Eriksson, K.-E. (1976), *Arch. Microbiol.* **109,** 1.
9. Tien, M. and Kirk, T. K. (1983), *Science* **221,** 661.
10. Glenn, J. K. and Gold, M. H. (1983), *Appl. Environ. Microbiol.* **45,** 1741.
11. Ishihara, T. (1980), In *Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications,* vol. 2, Kirk, T. K., Higuchi, T., and Chang, H. M., eds., CRC Press Inc., Boca Raton, FL, 17-32.

12. Higuchi, T. (1990), *Wood Sci. Technol.* **24,** 23.
13. Westermark, U. and Eriksson, K.-E. (1974), *Acta Chem. Scand.* **B28,** 209.
14. Eriksson, K.-E. (1990), *Wood Sci. Technol.* **24,** 79.
15. Ander, P., Mishra, C., Farrell, R. L., and Eriksson, K.-E. (1990), *J. Biotechnol.* **13,** 189.
16. Samejima, M. and Eriksson, K.-E. (1991), *FEBS Lett* **292,** 151.
17. Barrichelo, L. E. G. (1987), *Bol. Biotechnol., FEALQ (Brazil)* **8,** 2.
18. Auer, C. G., Ferrari, M. P., Tomazello Filho, M., and Barrichelo, L. E. G. (1987), *IPEF, Piracicaba, Brazil* **37,** 45.
19. Fengel, D. and Wegener, G. (1984), In *Wood: Chemistry, Ultrastructure, Reactions.* De Gruyter, Berlin, New York, 182–222.
20. Auer, C. G. (1986), MSC Dissertation, Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Piracicaba, Brazil.
21. DIFCO Manual (1978), DIFCO Laboratories, Michigan, USA, 245.
22. Szklarz, G. D., Antibus, R. K., Sinsabaugh, R. L., and Linkins, A. E. (1989), *Mycol.* **81,** 234.
23. Tien, M. and Kirk, T. K. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 2280.
24. Mandels, S. M., Andreotti, R., and Roche, C. (1976), *Biotechnol. Bioeng. Symp.* **6,** 21.
25. Miller, G. L. (1959), *Anal. Chem.* **31,** 426.
26. Pereira, H. and Sardinha, R. (1984), *Appita* **37,** 661.
27. Westermark, U. and Eriksson, K.-E. (1975), *Acta Chem. Scan.* **B29,** 419.
28. Dekker, R. F. H. (1980), *J. Gen. Microbiol.* **120,** 309.
29. Morpeth, F. F. (1985), *Biochem. J.* **228,** 557.
30. Kirk, T. K. and Shimada, M. (1985), In *Biosynthesis and Biodegradation of Wood Components.* Higuchi, T., ed., Academic, Orlando, FL, 579–605.
31. Kirk, T. K. and Farrell, R. L. (1987), *Ann. Rev. Microbiol.* **41,** 465.