

Laccase and Other Lignocellulose Modifying Enzymes of Marine Fungi Isolated from the Coast of India

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Abstract

Several genera of marine fungi isolated from the coast of India from decaying mangroves and seagrass were tested for the presence of laccase, one of the major lignin modifying enzymes present in many genera of wood-rotting fungi. Varying levels of laccase activity were present in most of the fungi included in this study. The laccase plate assay was found to be much less reliable than the spectrophotometric laccase using the extracellular culture fluid of the fungal cultures grown in liquid media. Several factors including type of growth medium, growth medium pH, and assay pH had marked effects on laccase activity. A few strains produced high levels of laccase in both malt extract (ME) medium and low N medium; however, a majority of the strains produced laccase in low N medium (pH 4.5) but not in the ME medium. When grown in low N medium at pH 4.5, twelve of the strains showed acidic laccase (pH 4.5) activity; of these, seven also showed alkaline laccase (pH 8.2) activity. One of the strains exhibited laccase activity only when grown in the low N medium at pH 8.2. Lignin peroxidase (LIP) was not present in any of the strains tested, but three of the strains showed manganese-dependent peroxidase (MNP) activity. Xylanase and cellulase activities were observed in a majority of the fungi studied. The results indicate that laccase is widely distributed in fungi found on decaying lignocellulosic materials in the marine environment and that a number of these fungi also contain other lignocellulose modifying enzymes, except LIPs and MNPs which appear to be relatively less common in these marine fungi.

Introduction

Marine fungi are often found on decaying lignocellulosic substrates such as proproots, pneumatophores, branches, leaves, and driftwood in the intertidal region of mangrove stands (Kohlmeyer and Kohlmeyer 1979). A majority of these fungi belong to Ascomycetes and Deuteromycetes, while Basidiomycetes are relatively rarely reported from such marine environments (Hyde and Jones 1988). Marine fungi are believed to be important in the degradation/mineralization of mangrove leaves, wood, seagrasses and other lignocellulosic materials resulting in the formation of detritus. Thus, the

fallen leaves and wood of mangroves are believed to be a significant primary source in the food web of the tropical marine ecosystem (Pomeroy 1980, Hodson and Moran 1989).

Mangrove leaves consist of about 50% lignocellulosic structural polymers and 50% soluble organics which include tannins and phenolics (Benner and Hodson 1985). Ascomycetes and Deuteromycetes, which are known to cause soft-rot decay of wood, are important wood degraders in aquatic environments as they require moist conditions and cause softening of the surfaces of the woody tissue. Several workers have shown that soft-rot fungal activity results in loss in weight of wood blocks and that it is targeted toward the syringylpropane units

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of lignin (Nilsson *et al.* 1989). Sutherland *et al.* (1982) demonstrated decomposition of ^{14}C -labeled maple and spruce lignins by a number of species of marine fungi. However, information to date has been sparse on the type of lignin-modifying enzymes present in marine Ascomycetes and Deuteromycetes (Cuomo *et al.* 1987, Rohrmann and Molitoris 1992).

During our studies on the role of fungi in detritus dynamics in mangrove swamps, we isolated several hyphomycetous and ascomycetous fungi from leaf litter and woody substrates in the mangroves. The objective of this study was to identify and screen these fungi for the presence of various lignocellulose-degrading enzymes with a particular focus on laccases, a major group of lignin modifying enzymes. Laccases along with lignin peroxidases (LIPs) and manganese-dependent peroxidase (MNP) constitute the three main classes of ligninolytic enzymes (Kirk and Farrell 1987, Boominathan and Reddy 1992, Higuchi 1993). Two or more of these three classes of enzymes are fairly widely distributed in the Basidiomycetes that cause white-rot decay of wood (Maltseva *et al.* 1991, Kantelinen *et al.* 1989, Vares *et al.* 1992, Coll *et al.* 1993, Reddy 1993), but there is very little information currently on the types of lignin-modifying enzymes present in various marine fungi. In this paper, we present evidence that laccase, which has been shown to be important in lignin degradation (Archibald and Roy 1992, Bourbonnais and Paice 1992, Higuchi 1993) and has important applications in the pulp and paper industry for bleaching of wood pulp (Paice *et al.* 1989, Bourbonnais and Paice 1992), is widely distributed in marine fungi. Moreover, xylanase, which has also been shown to have applications for bleaching of wood pulp (Viikari *et al.* 1986, Paice *et al.* 1988, Senior *et al.* 1991, Nakamura *et al.* 1993), is present in a majority of these laccase-positive marine fungi.

Material and Methods

Organisms and culture conditions

All the strains of fungi used in this study were isolated either from mangrove soils, decaying wood and leaves of various mangroves (from stands of *Rhizophora mucronata* Lamk., *Rhizophora apiculata* Blume and *Avicennia officinalis* Linn.) and leaves of the seagrass *Thalassia hemprichii* (Ehrenberg) Ascherson (as indicated in Table I) from the Andaman Islands (Bay of Bengal in the Indian Ocean, lat. $11^{\circ}35' \text{N}$ and long. $92^{\circ}36' \text{E}$), the coral islands of the Lakshadweep archipelago in the Arabian sea (long. $72^{\circ}-73^{\circ} \text{E}$, lat. $10^{\circ}30'-11^{\circ}0' \text{N}$), and from mangrove swamps of Chorao islands, Goa, India (long. $73^{\circ}55' \text{E}$ and lat. $15^{\circ}30' \text{N}$). Decaying leaves and soil samples were collected in sterile plastic bags and

processed further within 3 h. Discs of 0.5 cm diameter were cut from the leaves, surface sterilized in 0.5% sodium hypochlorite (Newell and Fell 1982), washed twice with 50 mL of filter-sterilized sea water and placed on plates of Poly-R 478 medium (Boominathan *et al.* 1990) supplemented with 10 000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 mL of medium (prepared with 50% sterilized seawater) to inhibit bacterial growth. The ability to decolorize Poly-R is believed to be positively correlated with an organism's ability to produce LIPs and/or MNPs (Boominathan and Reddy 1992). Therefore, Poly-R decolorizing colonies growing out from the leaf discs were isolated and maintained on plates of malt extract (ME) agar (20 g malt extract, 20 g glucose, and 1 g peptone, in 1 liter of distilled water, adjusted to pH 4.5 with HCl).

For isolating lignin-modifying obligate mangrove fungi, decaying wood pieces from mangrove swamps were incubated in the dark in sterilized boxes lined with moist filter papers for 7–10 days. Usually fruiting bodies appear on the surface of the decaying wood pieces within this period. Ascocarps were crushed in a drop of sterile sea water with a sterile scalpel and the ascospores were picked up by using a fine sterile glass capillary tube. The ascospores were then streaked on to corn meal agar (Difco Laboratories, Detroit, MI) medium made up with 50% seawater. Colonies arising from single ascospores were maintained on Boyd and Kohlmeyer (B & K) medium (Kohlmeyer and Kohlmeyer 1979) which contained in one liter of distilled water: 10 g glucose, 2 g peptone, 1 g yeast extract, 38 g synthetic sea salts (Instant Ocean of Aquarium Systems, Mentor, OH), and 18 g agar. The pH of the medium was adjusted to 7.5 with HCl. The ME medium (as described above) as well as a defined low nitrogen medium (low N medium) with acetate buffer (Dass and Reddy 1990) were used in our studies, except where otherwise mentioned. The low N medium contained the following per liter: 10 g glucose, 2 g KH_2PO_4 , 1.45 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.132 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg thiamine hydrochloride, 0.5 g Tween 80, 1.2 mM ammonium tartrate, 20 mM sodium acetate (pH 4.5), and 0.4 mM veratryl alcohol. The following trace elements were also added (per liter): 0.14 g nitrilotriacetate, 0.07 g NaCl, 0.007 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.033 g MnSO_4 , 0.013 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.007 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0011 g H_3BO_3 and 0.0007 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Since several fungi failed to grow in low N medium with acetate buffer, this buffer was replaced with 20 mM glycine-HCl buffer (pH 4.5) and the latter modification resulted in growth of these strains. The effect of salinity on growth of the marine fungal isolates was tested by adding synthetic sea salts to the ME medium to give 1.5% to 3.0% (w/v). Low N medium without veratryl alcohol and Tween 80 was used to test for laccase production. Low

N medium supplemented with 1% birchwood xylan (Sigma Chemicals, St. Louis, MO) was used to test for the production of xylanase.

Mycelial mats, grown in foam-plugged 125-mL flasks containing 20 mL ME medium for 7 d, were washed three times (100 mL each wash) with sterile distilled water, blended in low N medium using a Sorvall blender (with three 30-second spurts), and were used as inoculum. Low N medium (10 mL/125-mL flask) was inoculated with 1 mL of this inoculum (equivalent to about 3 mg dry weight of mycelium). The cultures were oxygenated immediately after inoculation and every third day thereafter. Oxygenation was accomplished by flushing oxygen over the cultures for 1 min at 5 p. s. i. using a sterilized cotton-plugged Pasteur pipette attached to an oxygen cylinder. In all cases 100% oxygen was used as lignin-modifying enzymes were known to be optimally expressed under 100% oxygen rather than in air (Kirk and Farrell 1987). All inoculations and oxygenations were done in a Laminar Air-Flow Hood (SterilGard Hood, The Baker Co. Inc., Maine) after sterilizing the interior with ultraviolet light. Incubation was at room temperature (22–23 °C) in static cultures as described previously by Dass and Reddy (1990). All cultures were routinely checked for purity by phase-contrast microscopy.

For determination of mycelial dry weight, duplicate cultures grown in ME medium were vacuum-filtered through tared Whatman GF/C filter papers, rinsed with 100 mL of distilled deionized water, dried to a constant weight, and the cell mass was calculated by difference.

Laccase plate assay

Laccase in plate cultures was detected using a modification of the method of Niku-Paavola *et al.* (1990). A 0.5 cm plug from the edge of an actively growing culture was transferred to Petri plates of ME or low N medium (pH 4.5) or low N medium with glycine-NaOH buffer (pH 8.2) containing 2 mM ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; Sigma Chemical Co, St. Louis, MO)]. Green or greenish-blue color under and around the fungal colonies was considered a positive reaction.

Laccase, LIP, and MNP enzyme assays

The fungus was grown in ME liquid or low N medium (10 mL/125-mL Erlenmeyer flask) as shallow liquid cultures without agitation under oxygen. The flasks were flushed with oxygen at the time of inoculation and again on day 3 of incubation as described above. Culture supernatants were assayed for laccase as described by

Niku-Paavola *et al.* (1988). The reaction mixture contained 14 μ M ABTS in 50 mM glycine-HCl (pH 4.5) or glycine-NaOH buffer (pH 8.2) in a final volume of 1.0 mL. The reaction was monitored by measuring the change in absorbance at 436 nm. The enzyme units were expressed as μ mol substrate transformed per minute per mL of culture supernatant. No increase in absorbance at 436 nm was observed in the absence of the enzyme or when boiled culture filtrate was used as controls. Lignin peroxidase (LIP) activity in extracellular culture fluid was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde as described by Teen and Kirk (1988). Manganese-dependent peroxidase (MNP) activity was determined using the method of Paszczyński *et al.* (1988).

Xylanase assay

Low N medium, without glucose, veratryl alcohol and Tween 80 but supplemented with 1% birchwood xylan (Sigma Chemicals, St Louis, MO), was used to test for the production of xylan. Culture supernatants from 7-day-old cultures were collected by centrifugation at 10 000 rpm for 10 min. Xylanase activity was assayed by measuring the amount of reducing sugars liberated from xylan substrate by the dinitrosalicylic acid method (Miller 1959). Briefly, 250 μ l of enzyme was added to 250 μ L of 0.5% birchwood xylan and 500 μ L of 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C for 1 h. The enzyme activity was terminated by adding 3 mL of dinitrosalicylic acid and the tubes were boiled at 100 °C for 10 min, cooled to room temperature, and the absorbance was measured at 640 nm. Xylanase activity is expressed as nmoles of reducing sugar released per minute per mL of culture supernatant under the assay conditions described above (Nakamura *et al.* 1993). Qualitative test of xylanase was carried out using 2% birchwood xylan as sole carbon source on plates of low N medium. Glucose, Tween 80 and veratryl alcohol were omitted from this medium. Clearance of xylan around xylanase positive fungal colonies results in appearance of a clear halo (Haack 1992).

Cellulase assay

The basal medium used here contained per liter: 15 g agar, 1 g yeast extract, 1.25 g KH_2PO_4 , and 0.625 g peptone. Cellulose azure overlay medium contained per liter: 15 g agar, 5 g malt extract, 1 g yeast extract, 1 g a-cellulose fibres, and 0.5 g Azure II (Sigma Chemicals). Cellulose azure tubes were prepared by dispensing 15 mL of basal medium into 25-mL screw-capped tubes, autoclaving at 121 °C for 20 min, allowing the tubes to cool in an upright position, and then aseptically dispens-

ing 0.8 mL of autoclaved overlay medium into each tube. Each tube was inoculated with a 5 mm disc cut from the margin of an actively growing colony on ME medium. Tubes were incubated at room temperature for up to 4 weeks. Cellulolysis is indicated by the release of the blue dye and its subsequent diffusion into the clear lower agar, whereas decolorization of the dye in the overlay medium indicates ligninolytic activity. These two activities could be monitored even when an organism has both LIP and cellulase, because decolorization of the cellulose azure normally follows diffusion of the dye into the lower agar layer (Thorn 1993).

Results

The fungi used in this study, isolation sources, general characteristics, and identification are presented in Table I. Of these *Gliocladium* sp. and *Sordaria fimicola* are terrestrial species which were isolated from mangrove soils, whereas *Aigialus grandis*, *Aigialus mangrovei*, *Lophiostoma mangrovei*, *Halosarpheia ratnagiriensis*, *Quintaria lignatilis*, *Verruculina enalia*, *Cirrenalia pygmaea*, *Zalerion varium* and *Hypoxylon oceanicum* are mangrove fungi, reported only from that habitat (Kohl-

meyer and Volkmann-Kohlmeyer 1991). Nine out of 17 fungi included in this study were Ascomycetes, 4 were Hyphomycetes and one was a Zygomycete. The taxonomic positions of strains 312, 315, and 316 are not clear at this time because they do not form spores.

Since all the fungi in this study were isolated from a marine environment, the effect of salinity on the growth of these organisms was tested in B & K medium for obligate marine species and in ME medium supplemented with Instant Ocean salts (at 1.5% or 3.0% w/v) for the others. Growth in unsupplemented ME and B & K media were used for comparison. The results showed dramatic improvement in growth of strains 298, 315, 318, and 328 in the presence of synthetic sea salts (Table II). Stimulation of growth in response to increased salinity was also seen with strains 321, 325 and 326 but the extent of growth stimulation was less marked.

Results of the laccase plate assay showed that many of the marine fungi included in this study exhibited laccase activity on plates of ME and low N medium (Table III). However, 5 of the 17 fungi (strains 289, 312, 319, 320, and 322) failed to give positive laccase reaction on plates of ME and low nitrogen medium.

Table I. Isolation sources, taxonomy, and growth characteristics of selected strains of fungi isolated from marine environments.

Strain	Genus	Taxonomic group	Source*	Growth characters on MEA plates
145	<i>Gliocladium</i> sp.	Hyphomycetes	B	Dark brown colony
303	<i>Gliocladium</i> sp.	Hyphomycetes	B	Dark brown colony
289	Unidentified	Ascomycetes	A	White and fluffy colony; forms immature cleistothecia
298	<i>Sordaria fimicola</i> (Roberge) Cesati et De Notaris	Ascomycetes	C	Dark brown colony
312	Unidentified	Basidiomycete (?)	D	White fluffy growth
313	<i>Gongronella</i> sp.	Zygomycetes	A	White fluffy colony
315	Unidentified	?	A	White mycelium with orange crystal-like structures
316	Unidentified	?	A	White fluffy colony
318**	<i>Aigialus grandis</i> Kohlmeyer et Schatz	Ascomycetes	E	Black colony
319**	<i>Aigialus mangrovei</i> Borse	Ascomycetes	E	Light brown mycelium; produces dark pink pigment
320**	<i>Lophiostoma mangrovei</i> Kohlmeyer et Vittal	Ascomycetes	E	Black colony
321**	<i>Halosarpheia ratnagiriensis</i> Patil et Borse	Ascomycetes	E	Brownish fluffy colony
322**	<i>Quintaria lignatilis</i> (Kohlmeyer) Kohlmeyer et Volkmeyer-Kohlmeyer	Ascomycetes	E	Dark brown colony
323**	<i>Verruculina enalia</i> (Kohlmeyer) Kohlmeyer et Volkmeyer-Kohlmeyer	Ascomycetes	E	Dark olive-green colony
325**	<i>Cirrenalia pygmaea</i> Kohlmeyer	Hyphomycetes	E	Black adpressed colony
326**	<i>Zalerion varium</i> Anastasiou	Hyphomycetes	E	White colonies
328**	<i>Hypoxylon oceanicum</i> Schatz	Ascomycetes	E	White fluffy colony

* A = Decaying mangrove leaves, Andaman islands, India; B = Mangrove soils, Andaman islands, India; C = Decaying mangrove leaves, Chorao island, Goa, India; D = Seagrass leaves, Lakshadweep islands, India; E = Decaying mangrove wood, Chorao island, Goa, India.

** Obligate marine fungi.

Table II. Effect of salinity on growth*.

	Organism	Strain	ME medium	B & K medium	Supplementation with IO	
					1.5%	3.0%
A	<i>Gliocladium</i> sp.	145	43.6	—	35.7	40.2
	<i>Gliocladium</i> sp.	303	28.4	—	30.9	36.7
	Ascomycete	289	67.8	—	60.4	69.6
	<i>S. fimicola</i>	298	5.5	—	65.9	105.4
	Unidentified	312	10.3	—	12.9	5.2
	Unidentified	315	6.7	—	58.7	7.9
	Unidentified	316	24.9	—	27.8	5.6
B	<i>A. grandis</i>	318	—	3.8	25.6	45.1
	<i>A. mangrovei</i>	319	—	24.8	27.8	24.3
	<i>L. mangrovei</i>	320	—	17.3	20.1	16.7
	<i>H. ratnagiriensis</i>	321	—	52.1	108.8	124.5
	<i>Q. lignatilis</i>	322	—	17.9	16.2	26.7
	<i>V. enalia</i>	323	—	21.7	5.0	19.1
	<i>C. pygmea</i>	325	—	18.1	24.9	52.0
	<i>Z. varium</i>	326	—	28.9	42.0	28.1
	<i>H. oceanicum</i>	328	—	7.8	40.1	53.1

* Growth values are given as mg mycelial dry wt per 20 mL of a given medium. Instant Ocean salts (IO), described in Materials and Methods, were added at the concentrations indicated to ME medium (section A) or to B & K medium (section B). '—' indicates Not Tested.

Quantitative measurements of laccase activity using extracellular fluid of cultures grown in ME or in low N liquid medium were done to confirm results of the plate assay. The results showed inconsistencies between the plate assay (Table III) and the spectrophotometric laccase assay (Table IV). For example, only six of the seventeen fungal strains tested positive for laccase activity when grown in ME liquid medium whereas 12 strains

tested positive for laccase on ME plates (Tables III and IV). Five strains which tested negative for laccase activity on ME plates (*i.e.* strains 289, 312, 319, 320, and 322) also tested negative for the spectrophotometric laccase assay. Our results further showed that laccase production by a number of fungi is suppressed in ME liquid medium, which is complex and nutritionally rich and therefore may be less suitable than defined low N medium for studying laccase production by marine fungi. This is illustrated by the fact that eight of the strains which tested negative for laccase activity when grown in ME liquid medium, did test positive for laccase when grown in low N medium (Table IV). Eleven out of the fifteen fungi that tested positive for laccase in low N medium showed higher activity when grown and assayed at low pH (4.5) but showed little or no activity at alkaline pH (8.2). It was of interest that those strains that appear to be well adapted to the marine environment, as shown by considerable stimulation of their growth in the presence of synthetic sea salts (*e.g.*, strains 298, 315, 318, 321, and 326), gave laccase activity both at acidic and alkaline pH. The pH of 8.2 was chosen to approximate the pH of the sea water.

Strain 315, which showed about 8-fold increase in growth in the presence of the synthetic sea salts (see Table II), failed to show laccase activity when grown in low N medium at pH 4.5, but showed laccase activity when grown in low N medium at pH 8.2. However, it was of interest that laccase, even in the strains grown at pH 8.2, was most active when tested at pH 4.5. This observation is consistent with a recent report of Coll *et al.* (1993) who showed that a basidiomycete strain PM1

Table III. Laccase activity of marine fungi.

Organism	Strain #	Laccase plate assay*	
		MEA (pH 4.5)	Low N (pH 4.5)
<i>Gliocladium</i> sp.	145	+++	+++
<i>Gliocladium</i> sp.	301	+++	++
Ascomycete	289	—	—
<i>S. fimicola</i>	298	++	+
Unidentified	312	—	—
<i>Gongronella</i> sp.	313	+++	+
Unidentified	315	+++	+
Unidentified	316	+	+
<i>A. grandis</i>	318	++	+
<i>A. mangrovei</i>	319	—	—
<i>L. mangrovei</i>	320	—	—
<i>H. ratnagiriensis</i>	321	+++	+++
<i>Q. lignatilis</i>	322	—	—
<i>V. enalia</i>	323	+	++
<i>C. pygmea</i>	325	+++	+++
<i>Z. varium</i>	326	++	—
<i>H. oceanicum</i>	328	+	++

* Malt extract agar (MEA) and low N medium are described in Materials and Methods. Laccase activity is based on oxidation of the colorless redox indicator 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate) to a blue-green color. Relative amounts of laccase production: +++, strong activity; ++, moderate activity; +, low activity; and —, no activity.

Table IV. Laccase activity in ME and low N media under acidic and alkaline conditions*.

Organism	Spectrophotometric laccase assay					
	ME medium (pH 4.5)		Low N medium (pH 4.5)		Low N medium (pH 8.2)	
	Assayed at pH 4.5	Assayed at pH 8.2	Assayed at pH 4.5	Assayed at pH 8.2	Assayed at pH 4.5	Assayed at pH 8.2
<i>Gliocladium</i> sp. #145	51.2	ND	33.6(5)	2.7(3)	2.7(3)	ND
<i>Gliocladium</i> sp. #303	3.9	ND	30.0(7)	3.1(3)	3.1(3)	ND
Ascomycete #289	ND	ND	4.1(5)	ND	ND	ND
<i>S. fimicola</i> #298	73.1	ND	10.0(7)	6.8(7)	31.4(3)	13.0(7)
Unidentified #312	ND	ND	4.6(5)	5.5(7)	1.4(5)	2.7(5)
<i>Gongronella</i> sp. #313	ND	ND	1.4(5)	ND	ND	ND
Unidentified #315	ND	ND	ND	ND	6.8(5)	2.3(5)
Unidentified #316	ND	ND	5.5(7)	ND	ND	ND
<i>A. grandis</i> #318	ND	ND	1.4(5)	ND	10.3(4)	ND
<i>A. mangrovei</i> #319	ND	ND	ND	ND	ND	ND
<i>L. mangrovei</i> #320	ND	ND	17.1(5)	ND	28.7(4)	ND
<i>H. ramagiriensis</i> #321	8.9	ND	12.3	2.7	4.1(4)	ND
<i>Q. lignatilis</i> #322	ND	ND	NT	NT	ND	ND
<i>V. enalia</i> #323	14.3	ND	ND	ND	1.4(4)	ND
<i>C. pygmea</i> #325	1.4	ND	18.2	4.1	ND	ND
<i>Z. varium</i> #326	ND	ND	9.5	4.7	8.9(5)	ND
<i>H. oceanicum</i> #328	ND	ND	ND	2.9	NT	NT

* The organisms were grown in malt extract liquid medium (ME medium; pH 4.5) and in low N medium (Dass and Reddy 1990) from which veratryl alcohol and Tween 80 were deleted and acetate buffer was replaced with 20 mM glycine-NaOH buffer (pH 8.2) or 20 mM glycine-HCl buffer (pH 4.5). Spectrophotometric laccase assay was based on oxidation of ABTS as described in Materials and Methods. Laccase units are presented as $\mu\text{mol min}^{-1} \text{mL}^{-1}$. The numbers in parentheses represent the day of maximum activity. ND = not detected; NT = not tested.

produced laccase activity that was stable from pH 3.0 to 9.0 with an optimal pH at 4.5.

Activity of LIP was not detected in any of the cultures but three cultures (strains 298, 312, and 321) showed MNP activity. The first two strains produced MNP in low N medium buffered with acetate buffer, whereas strain 321 did not grow in this medium. However, the latter strain did grow in low N medium buffered with glycine-HCl buffer (pH 4.5) and showed MNP activity (390 U/L on d 15).

As xylanases have been shown to be potentially important for bleaching of paper pulps and for other industrial applications (Viikari *et al.* 1986, Buchert *et al.* 1992) selected fungal strains with laccase activity and desirable growth characteristics were tested for xylanase activity (Table V). Cellulolytic and ligninolytic activities in these strains, as detected by the cellulose azure assays, is also presented for comparative purposes. The results showed that most of the strains tested have xylanase activity, albeit at low levels, except for strains 313 and 319 which have fairly high levels of xylanase. Most of the xylanase-positive strains also showed cellulolytic and ligninolytic activity (Table V).

Discussion

There is a wealth of information available on the extracellular ligninolytic enzymes of the white-rot fungi in-

cluding LIPs, MNPs, and laccase but information on the ligninolytic enzymes of soft-rot fungi has been very limited to date (Kirk and Farrell 1987, Buswell and Odier 1987, Boominathan and Reddy 1992, Higuchi 1993, Reddy 1993). Ascomycetes are commonly associated with decaying mangrove leaves and seagrasses in the marine environment (Kohlmeyer and Kohlmeyer 1979). Previous studies have reported on the presence of lignocellulose degrading ability in some of these fungi (Gessner 1980, Sutherland *et al.* 1982, Cuomo *et al.* 1987, Bergbauer and Newell 1992). Our results show the presence of a number of known lignocellulose modifying enzymes in various genera of the marine fungi included in this study.

Independent studies have demonstrated that LIPs, MNPs, and laccases are capable of modifying lignin (Galliano *et al.* 1988, Kirk and Farrell 1987, Archibald and Roy 1992, Bourbonnais and Paice 1992, Higuchi 1993). While a few white-rot fungi such as *Trametes versicolor* (Linnaeus : Fries) Pilat produce all three classes of enzymes, many of the white rot fungi studied so far appear to have the ability to produce only one or two families of the above mentioned enzymes (Bollag and Leonowicz 1984, Kirk and Farrell 1987, Galliano *et al.* 1988, Maltseva *et al.* 1991, Rüttimann *et al.* 1992, Vares *et al.* 1992). Our results show that three of the marine fungal isolates studied produced MNPs and lac-

Table V. Xylanolytic, cellulolytic and ligninolytic activities.

Organism	Xylanase* (nmol min ⁻¹ ml ⁻¹)	CEA Cellulolytic**	CEA Ligninolytic**
<i>Gliocladium</i> sp. #145	40.9	+	+
Ascomycete #289	48.5	-	-
<i>S. fimicola</i> #298	50.1	+	+
Unidentified #312	46.8	+	+
<i>Gongronella</i> sp. #313	574.0	-	+
<i>A. grandis</i> #318	58.5	+	+
<i>A. mangrovei</i> #319	222.0	+	+
<i>L. mangrovei</i> #320	103.6	+	+
<i>H. ratnagiriensis</i> #321	23.4	+	+
<i>Z. varium</i> #326	33.4	+	+
<i>H. oceanicum</i> #328	177.0	+	+

* To test for xylanase activity, the organisms were grown in low N medium (pH 4.5) containing 1% birch-wood xylan as the sole carbon source. The assay procedure is described in Materials and Methods. The values represent the maximum enzyme activity seen with each culture.

** To test for cellulolytic and ligninolytic activities, the organisms were inoculated into cellulose azure agar (CEA) tubes as described in Materials and Methods. Cellulolytic and ligninolytic activities are shown as (+) = positive, or (-) = negative.

cases (strains 298, 312, and 321) while a majority produced only laccases.

Laccases (EC 1.10.3.2), copper-containing glycoproteins commonly found in wood-decay fungi (Reinhammar 1984, Leonowicz *et al.* 1985, Mayer 1987), have been implicated in fungal degradation of lignin and its derivatives (Galliano *et al.* 1988, Archibald and Roy 1992, Higuchi 1993). Laccases have been reported to depolymerize lignins and various lignin model compounds (Archibald and Roy 1992, Bourbonnais and Paice 1992, Higuchi 1993, Galliano *et al.* 1988). Both inducible and constitutive laccases have been reported from fungi (Bollag and Leonowicz 1984, Rogalski *et al.* 1990). Our results show that the fungi tested in this study produced constitutive laccase in low N medium in the absence of any known inducers such as veratric acid or other aromatic compounds. The presence of both acidic and alkaline laccases in some of the marine fungi and the fact that one organism produced laccases only under alkaline conditions is also of interest. The presence of laccases in a number of marine fungi tested suggests that these organisms play a role in lignin modification in the marine environment. The observation that all the fungi exhibited higher laccase activity when assayed at pH 4.5, suggest that these laccases may operate best at acidic microenvironments in nature.

Laccase plate assays are easy, quick, and convenient (Sundman and Nase 1971, Niku-Paavola *et al.* 1990), and are based on the ability of an organism to oxidize compounds such as ABTS, guaiacol, and syringaldazine incorporated in the media resulting in intense color production next to the fungal colony. However, in a number of instances the results of laccase plate assay were not substantiated by the quantitative laccase assays. Our results also point to the need (at least with marine fungi) to

use extracellular fluids from cultures grown in nitrogen-limited media at both acidic and alkaline pH values before drawing conclusions about the laccase producing ability of a given organism.

It has been well established that ligninolytic enzyme systems in many white-rot basidiomycetes are produced in response to starvation for nutrients such as N and C and that excess N and C strongly repress these enzyme systems (Kirk and Farrell 1987, Reddy 1993). In this study (Table III) we observed that a number of the marine fungi tested did not exhibit laccase production in ME medium but did so in low N medium. Further results also showed that strain 312 did not produce laccase in high N medium (unpublished data). Taken together, these data indicate that laccase production in marine fungi, similar to LIP and MNP production in white-rot fungi, is suppressed by high levels of nitrogen and/or other nutrients systems (Kirk and Farrell 1987, Reddy 1993). However, it is of interest that strains 145 and 298 (and to a lesser extent, 321 and 323) produced high levels of laccase in both ME medium as well as in the low N medium. Strain 298 is particularly noteworthy in that it produced about 7-fold greater laccase activity in ME medium as compared to that in low N medium.

Viikari and co-workers (1986) suggested that xylanases can be used to enhance the bleaching of kraft pulps and several workers have shown the potential of xylan degrading fungi for use in bio-bleaching of wood pulp (Paice *et al.* 1988, Senior *et al.* 1991, Buchert *et al.* 1992). In view of this, selected strains of marine fungi were screened in this study for the production of xylanase. A majority of the fungi tested had varying degrees of xylanase activity.

Chrysonilia sitophila Mont von Arx (Durán *et al.* 1987), an Ascomycete fungus, has been reported to produce

multiple LIPs and no MNPs. Our studies show that two of the ascomycetous fungi (strains 298 and 321) and an unidentified fungus (strain 312), included in this study, produced MNP activity in low N medium. To the best of our knowledge these findings represent the first report of MNPs in marine fungi. It was of interest that the MNP activity in strains 298 and 321 was seen only after 15 days of inoculation, several days after their growth maxima had been reached (data not shown), whereas in the widely-studied white-rot fungus, *Phanerochaete chrysosporium* Burds, MNP activity reached a peak within 4–6 days after inoculation (Kirk and Farrell 1987, Reddy 1993). Further work on the characterization of the MNPs in these marine fungi is in progress.

Research on ligninolytic microorganisms and their enzymes has intensified in recent years because of their many potential industrial applications (see Boominathan and Reddy 1992). In view of this, screening of marine fungi as a potential source for interesting and unusual lignocellulolytic enzymes of practical value has been in-

itiated. This study has yielded several interesting fungal strains that produced varying combinations of laccase, MNP, xylanase, and cellulase activities. From an ecological standpoint, these data indicate the potential importance of marine fungi (individually and collectively) in lignocellulose digestion in the marine environment. Furthermore, the results show that a number of marine fungi produce both acidic and alkaline laccases that appear interesting on the basis of their scientific novelty as well as their potential for practical applications.

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