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Extracellular enzyme production by freshwater ascomycetes

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Thirty species of freshwater ascomycetes isolated from woody and/or herbaceous substrates were screened for their ability to produce extracellular degradative enzymes on solid media. Enzymes tested included: amylase, endoglucanase, endoxylanase, ß-glucosidase, laccase, lipase, pectinase, peroxidase, polygalacturonase, polyphenoloxidase, protease, tyrosinase and ß-xylosidase. All species were positive for cellulase and endoxylanase/ß-xylosidase. Two species, *Chaetomastia typhicola* (herbicolous) and *Massarina* sp. A25 (lignicolous) tested positive for all enzyme assays. *Submersisphaeria aquatica* (lignicolous) was positive for all enzymes except tyrosinase and *Jahnula* sp. A322 (lignicolous) was positive for all enzymes except polyphenoloxidase. Generally, the species which were isolated from herbaceous substrates and woody/herbaceous substrates had good growth rates on different types of enzyme media used (such as, peptone, yeast extract, glucose agar, etc.). Fifty percent of the lignicolous species produced pectin degrading enzymes, compared to about 80% for herbicolous and woody/herbicolous species, suggesting that there may be some specialization in the types of enzymes produced within substrate groups. The greatest differences among species occurred in the production of enzymes associated with detection of lignin degradation. Laccase and peroxidase detection depended on the assay technique used. Freshwater ascomycetes, as a group, produce many of the extracellular enzymes important in the decomposition of plant structural materials thereby supporting the idea that they play an important role in recycling in aquatic habitats.

Key words: aquatic, decomposition, fungi, woody debris.

Introduction

Freshwater ascomycetes occur commonly on a wide variety of dead submerged plant substrates such as wood or emergent macrophytes (Shearer, 1993, 2001). With the exception of studies by Zare-Maivan and Shearer (1988 a,b) and Yuen *et al.* (1998) little is known about the enzymatic capabilities and decomposition activities of these fungi. In contrast, much more is known about this aspect in the freshwater 'Ingoldian' or aquatic hyphomycetes that colonize submerged deciduous leaves and woody debris (Chamier, 1985; Abdel-

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Raheem, 1997). These fungi produce a wide range of plant cell wall degrading enzymes (Chamier, 1985) and are able to cause weight loss in leaves (Triska, 1970; Bärlocher and Kendrick, 1974; Suberkropp and Klug, 1976, 1981; Chamier and Dixon, 1982; Suberkropp *et al.*, 1983; Zemek *et al.*, 1985; Butler and Suberkropp, 1986; Abdel-Raheem and Badran, 1997), and wood (Sanders and Anderson, 1979; Abdel-Raheem, 1997).

Among the freshwater ascomycetes, substrate specialization with respect to broad categories (herbaceous vs. woody) occurs (Shearer, 2001), i.e. most species are found on herbaceous or woody substrates, but only a few species are found on both. Whether or not differences in enzymatic capabilities exist among species from different substrate specific groups is not known.

The following study was undertaken to determine: (1) the kinds of enzymes produced by freshwater ascomycetes that could play a role in plant decomposition, and (2) whether there are qualitative differences in the enzymes produced among lignicolous, herbicolous and substrate non-specific freshwater ascomycetes.

Materials and methods

Thirty ascomycete species isolated from submerged plant substrates in the USA were selected for study (Table 1): eight species have been reported only from herbaceous substrates, 14 species only from woody substrates, and eight species from both woody and herbaceous debris (Shearer, 2001). All cultures were isolated according to the procedures of Shearer (1993) and Shearer *et al.* (2002). Strains are maintained in the culture collection of C.A. Shearer.

Species were grown on peptone, glucose, yeast extract agar (PYG) that provides amino acids, a simple carbon source, and vitamins, respectively (peptone 1.25 g, glucose, 3 g, yeast extract 1.25 g, agar 18 g and one liter of de-ionized water). Inoculum discs (5 mm diam.) were cut from the leading edge of colonies grown on PYG (30 ml in 9 cm plastic Petri dishes).

Production of extracellular enzymes was determined by incorporation of test substrates into a basic medium, inoculating the medium with discs of fungal hyphae, allowing the fungi to grow out on the medium, and adding reagents to the plates to detect the test substrate remaining. Colony radial growth rates and substrate clearing zones were measured for each fungal species on each substrate. Three replicates of each treatment were assayed and non-inoculated plates with substrates served as negative controls. Inoculated plates were checked at 5 or 10 days depending on the growth rates of the individual species. When the colonies had grown to 50-60% of the area of the

Table 1. Fungal strains used in this study with, isolate number, and substrate isolated from which they were isolated.

Species	Authorities	Strain	Substrate
Ascovaginospora stellipala	Fallah & Shearer	P5-1E	Herbaceous
Chaetomastia typhicola	(Karst.) Leuchtmann	A6-ID	Herbaceous
Massariosphaeria scirpina	(Winter) Leuchtmann	A8-2D	Herbaceous
Massariosphaeria sp. A-33		A33-2C	Herbaceous
Ophioceras arcuatisporum	Shearer, J.L. Crane & W. Chen	A167-1C	Herbaceous
Phaeosphaeria typharum	(Desm.) Holm	A165-ID	Herbaceous
Pleospora sp. A168-1		A168-IE	Herbaceous
Pyrenomycete sp. A90-1		A90-1B	Herbaceous
Annulatascus velatisporus	K.D. Hyde	A70-1B	Woody
Boerlagiomyces websteri	Shearer & J.L. Crane	A59-1B	Woody
Byssothecium flumineum	J.L. Crane, Shearer & Huhndorf	A21-3C	Woody
Discomycete sp. A93		A93-2A	Woody
Halosarpheia heteroguttulata	C.W. Wong, K.D. Hyde & E.B.G. Jones	A108-7C	Woody
Jahnula sp. A322		A322	Woody
Kirschsteniniothelia elaterascus	Shearer	A22-1A	Woody
Lasiosphaeria ovina	(Pres.:Fr.) Cesati & de Not.	A-120-1A	Woody
Loculoascomycete sp. A242		A242-3E	Woody
Massarina sp. A25		A25-1B	Woody
Massarina ingoldiana	Shearer & K.D. Hyde	A39-1B	Woody
Pseudohalonectria sp. A251		A251-1D	Woody
Pseudoproboscispora caudae-suis	(Ingold) J. Campb., Shearer, J.L. Crane & Fallah	A40-11B	Woody
Submersisphaeria aquatica	K.D. Hyde	A354-1D	Woody
Cercophora newfieldiana	(Ellis & Everh.) R. Hilber	A136-1A	Woody & herbaceous
Hymenoscyphus scutula	(pers.:Fr.) Philips var. furcata Philips	A1-1A	Woody & herbaceous
Loculoascomycete sp. A211		A211-5B	Woody & herbaceous
Ophioceras commune	Shearer, J.L. Crane & W. Chen	A165-1D	Woody & herbaceous
Phomatospora sp. A5		A5-1B	Woody & herbaceous
Pyrenomycete sp. A3-1		A3-1B	Woody & herbaceous
Pyrenomycete sp. A54		A54-11F	Woody & herbaceous
Pyrenomycete sp. A161		A161-1B	Woody & herbaceous

plate, chemical indicators were added to assay enzyme activity and activity zones were measured when appropriate.

Amylase

Amylase activity was assayed by growing the fungi on starch medium (starch – 2 g, peptone – 1 g, yeast extract – 1 g, agar – 20 g; distilled water – 1 L. After 5-10 days, the plates were flooded with a 1% aqueous IKI solution. A yellow zone around the colony in an otherwise blue medium was considered a positive test for starch hydrolysis (Gessner, 1980).

Lipase

Lipase activity was determined by growing the isolates on a medium containing a lipid (Tween 20, Sigma Chemical Co.) as the primary source of carbon. The medium was as follows: peptone – 1 g, yeast extract – 0.1 g, agar – 18 g, Tween 20 – 10 mL (autoclaved separately from the rest of the medium), distilled water – 990 mL. A positive test was the occurrence of precipitated fatty acid crystals around the colony (Gessner, 1980).

Pectolytic enzymes

To detect pectylotic activity, we used the medium described by Hankin and Anagnostakis (1975). This medium contained 500 mL of mineral salt solution, 1 g yeast extract, 15 g of agar, 5 g of pectin, and 500 mL of distilled water. The mineral salts solution contained per liter: 2 $(NH4)_2$ SO4, 4 g KH2 PO4, 6 g Na2 HPO4, 0.2 g FeSO4.7 H_2O, 1 mg CaCl2, 10 µg H3BO3, 10 µg Mn SO4, 10 µg ZnSO4, 50 µg CuSO4, 10 µg; MoO3, pH7 or pH $_5$ as needed. This medium at pH7 was used to detect pectate lyase production. The same medium at pH5 was used to detect polygalacturonase activity. For all tests, plates were incubated for 5-10 days and then flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. This reagent precipitates intact pectin in the medium and thus a clear zone around a colony in an otherwise opaque medium indicates degradation of the pectin.

Proteolytic enzymes

The medium used to detect proteolytic enzyme activity contained gelatin as the protein substrate (Hankin and Anagnostakis, 1975). This medium consists of nutrient agar plus 0.4% gelatin at pH6. An 8% solution of gelatin in water was sterilized separately and added to the nutrient agar at the rate of 5 mL per 100 mL of medium. After incubation, plates were flooded with an aqueous saturated solution of ammonium sulfate which precipitates protein. A clear zone around colonies indicated the presence of protease.

Cellulolytic enzymes

Three methods were used to detect cellulolytic enzymes (cellulase, endoglucanase and ß-glucosidase). These methods were described in detail by Pointing (1999). The cellulolysis basal medium (CBA) consisted of: $C_4H_{12}N_2O_6$ – 5 g, KH_2PO_4 – 1 g, $MgSO_4.7H_2O$ – 0.5 g, yeast-extract – 0.1 g, $CaCl_2.2H_2O$ – 0.001 g, distilled water – 1L.

Cellulase (cellulose azure agar)

CBM medium supplemented with 1.8% w/v agar was transferred in 10 mL aliquots to glass culture bottles, autoclaved, and allowed to solidify. Gently and carefully mixed viscous 0.1 mL CBM medium supplemented with 1% w/v cellulose azure (azure I dye, C.I.52010) and 1.8% w/v agar was added to the surface of the solidified agar as an overlay. The BI-layered medium was inoculated with discs of the test fungi and incubated. Migration of dye into the clear lower layer indicated the presence cellulase (Pointing, 1999).

Endoglucanase (CMC agar)

CBM medium was supplemented with 1% w/v low viscosity carboxymethylcellulose (CMC) and 1.8% w/v agar was added. The medium was autoclaved, dispensed into Petri dishes, allowed to solidify and inoculated with discs of the test fungi and incubated. After growth for 5-10 days, the plates were flooded with 2% aqueous Congo red (C.I.22120) and allowed to sit for 15 minutes. The stain was washed from the agar surface with distilled water and the plates were then flooded with 1 M NaCl to destain for 15 minutes. The NaCl solution was then removed. CMC degradation around the colonies appears as a yellow-opaque area against a red color for un-degraded CMC (Pointing, 1999).

ß-glucosidase (Esculin agar)

Activity of ß-glucosidase was detected by growing the test fungus on agar containing esculin (6,7-dihydroxycomarin-6-glucosidase) as the sole carbon source. CBM medium was supplemented with 0.5% esculin, 1.8% w/v agar and autoclaved. One mL of a sterile 2% w/v aqueous ferric sulphate solution was aseptically added for each 100 mL CBM prepared. The medium was dispensed into Petri dishes, allowed to solidify, inoculated and incubated. A black color develops in the medium of colonies producing ß-glucosidase (Pointing, 1999).

Hemicellulolytic (xylanolytic) enzymes

Two methods were used to detection xylanolytic enzyme activity: dye staining of xylan agar and dye diffusion from a xylan-dye complex [remazol

brilliant blue R bound to a modified (soluble) xylan (4-O-methyl-D-glucourono-D-xylan)]. These methods were described in detail by Pointing (1999).

Lignin enzymes

The process of lignin degradation is brought about by a suite of oxidative enzymes including polyphenol oxidase, tyrosinase, peroxidase, and laccase. The production of these enzymes was assayed using the detailed methods presented by Pointing (1999). Peroxidase production also was determined by a modification of the procedures of Egger (1986). Fungi were grown on CMA in Petri dishes for 5-10 days. Wells approximately 5 mm in diam. were cut in the test medium near the growing edges of colonies. One drop each of freshly prepared 1% w/v aqueous solution of pyrogallic acid and 0.4% hydrogen peroxidase activity was added to each well; presence of a golden yellow to brown color indicates peroxidase activity.

Results

Generally, species from herbaceous substrates and woody/herbaceous substrates had the fastest growth rates on PYG and most of the test substrates (Table 2). The least growth generally occurred on the peroxidase 1 and laccase 2 media of Pointing (1999). Otherwise, all species grew to some degree on most test substrates. All species were positive for cellulase, endoxylanase and ß-xylosidase (Tables 3, 4). Most species were able to degrade, to some degree, starch, lipid, protein, pectin, and hemicellulose and produced enzymes thought to be active in lignin decomposition such as polyphenoloxidase, peroxidase, tyrosinase and laccase (Tables 3, 4). Two species, *Chaetomastia typhicola* (herbicolous) and *Massarina* sp. A25 (lignicolous) were able to degrade all substrates tested. *Submersisphaeria aquatica* (lignicolous) was positive for all enzymes except tyrosinase and *Jahnula* sp. A322 (lignicolous) was positive for all enzymes except polyphenoloxidase.

Species differed mostly in their ability to produce lignin-degrading enzymes (Table 3). Twenty-one species were positive for polyphenoloxidase, while only 16 species were positive for tyrosinase. Peroxidase activity of the species differed according to the method used. For azure B agar (PE1) (Pointing, 1999), only five species tested positive, while for the second method (PE2) (Egger, 1986), twenty species tested positive. All species except *Ascovaginospora stellipala* were positive for laccase using the ABTS method (LA1) (Pointing, 1999), while only four species were only positive for laccase using the syringaldazine well test (LA2) (Egger, 1986).

Discussion

Solid media enzyme assays detect enzyme synthesis, release from the mycelium, and activity in the medium following production. Thus the lack of a positive result could mean that either the enzyme is not produced, or that it is produced and not released from the mycelium, or that it is produced and released, but the medium inhibits its detection. Thus absence of a reaction is not absolute confirmation of a species inability to produce a particular enzyme.

The relationship between the ability to grow on a particular test medium and ability to produce the corresponding enzyme to digest the substrate incorporated into the medium is not well correlated. All species in this study grew reasonably well on most assay substrates except for PE1 (Pointing, 1999) and LA2 (Egger, 1986). Most species however, did not produce enzymes on all the media on which they grew. Positive growth but negative enzyme results could be due to the reasons discussed in the preceding paragraph, the ability of the fungus to use other materials in the medium rather than the test substrate, or the fungus growing solely from the carbon source in the inoculum discs. In some cases, a fungus may grow rapidly across a plate when conditions are unfavorable, for example on plain water agar. Growth rates of the fungal species in this study were similar to those reported for tropical freshwater fungi (Yuen *et al.*, 1998). Growth rates of herbicolous and lignicolous/herbicolous species were slightly higher than those of lignicolous species. Whether this is a reflection of the longevity of their respective substrates, i.e. fungi on more rapidly decomposed substrates may have faster growth rates, is not known.

Pectic substances function as intercellular cement between plant cells, and degradation of these substances causes cell separation, which decreases the size of detritus and increases the surface area available to decomposers. A number of enzymes are involved in the hydrolysis of pectic substances. The activity of two of these complexes was detected in this study, Polygalacturonase (Dingle *et al.*, 1953) cleaves the 1, 4 glycosidic bonds between uronic acid monomers, and pectin methyl esterase (McComb and McCready, 1958) removes the methoxyl groups to yield pectic acid. The extracellular pectic enzyme polygalacturonase and pectate lyase are active at pH5 and pH7, respectively. Media at pH7 will not detect galacturonase and vice versa (Hankin *et al.*, 1971). Pectins are diluted to quantitative insignificance during secondary thickening and lignification of the cell wall of wood tissue (Kirk, 1983), therefore, the possession of enzymes necessary to degrade pectins may not be critical to the success of wood colonizing fungi. Zare-Maivan and Shearer (1988b) found support for this idea when they found that three commonly occurring species known only from submerged wood, *Leptoshaeria* sp., *Nais inornata* and *Trichocladium lignicola*, lacked pectinases

Table 2. Colony diam. (cm) after growth for ten days on the different test media.*

Species	Media																
	PYG	AM	PR	LP	PEC	PG	CL	EN	ßG	EX&BX1	EX&BX2	PX	PE1	PE2	TY	LA1	LA2
Ascovaginospora stellipala	3.9	5.3	3.7	2.6	7.0	5.8	2.8	5.1	4.1	3.6	3.6	3.6	-	3.6	3.3	0.2	-
Chaetomastia typhicola	2.0	2.2	2.2	1.7	1.7	1.9	1.9	2.5	3.1	2.1	1.7	2.5	-	1.9	1.7	1.7	-
Massariosphaeria scirpina	1.6	1.8	2.3	1.3	1.7	1.7	0.9	1.4	0.7	1.0	1.0	1.6	-	1.0	1.0	0.7	-
Massariosphaeria sp. A-33	3.9	7.0	3.4	3.6	2.9	3.2	2.5	6.3	6.7	6.7	5.2	8.0	-	5.6	6.2	5.0	-
Ophioceras arcuatisporum	3.6	2.2	3.2	3.3	3.0	2.2	1.9	0.5	0.6	2.9	2.8	3.7	-	2.9	3.2	2.2	-
Phaeosphaeria typharum	4.4	4.3	2.5	2.6	2.9	3.8	1.4	4.8	3.7	2.9	2.4	3.0	-	2.5	2.3	1.6	-
Pleospora sp. A168-1	2.6	2.5	2.9	2.8	2.2	2.6	1.2	1.2	0.6	1.1	3.6	1.4	-	-	-	1.2	-
Pyrenomycete sp. A90-1	0.8	0.8	0.9	0.7	1.4	1.5	0.9	0.8	-	0.9	0.8	0.8	-	1.5	1.5	1.1	-
Annulatascus velatisporus	1.1	1.7	2.4	1.1	2.1	2.6	1.4	2.1	2.2	1.4	1.5	2.2	-	1.7	1.7	1.3	-
Boerlagiomyces websteri	1.6	0.7	0.6	0.8	1.3	0.9	0.6	1.0	1.5	1.2	1.2	0.7	-	1.4	1.4	0.7	-
Byssothecium flumineum	1.6	1.5	1.8	1.4	1.4	1.6	1.0	0.8	1.0	0.9	0.9	1.9	-	1.1	1.1	1.1	-
Discomycete sp. A-93	1.2	1.6	1.0	1.5	1.1	0.9	1.4	1.0	0.6	0.7	0.9	3.6	-	1.1	1.2	0.9	-
Halosarpheia heteroguttulata	1.2	1.2	1.2	0.9	0.6	0.9	0.6	0.7	0.6	0.6	1.0	0.7	-	0.9	-	0.6	-
Jahnula sp. A322	1.4	1.4	1.8	1.6	1.7	1.4	0.7	1.1	2.8	1.4	0.7	1.3	-	0.9	0.8	1.1	2.2

* PYG: yeast extract glucose agar; AM: amylase; PR: protease; LP: lipase; PEC: pectinase; PG: polygalacturonase; CL: cellulase; EN: endoglucanase; ßG: ß-glucosidase; EX&BX1: Endoxylanase & ß-xylosidase (Method 1; Pointing, 1999); EX&BX2: endoxylanase & ß-xylosidase (Method 2; Pointing, 1999); PX: polyphenoloxidase; PE1: peroxidase (Pointing, 1999); PE2: peroxidase (Egger, 1986); TY: tyrosinase; LA1: laccase (Pointing, 1999); LA2: laccase (Egger, 1986).

Table 2. (continued).

Species	Media																
	PYG	AM	PR	LP	PEC	PG	CL	EN	ßG	EX& BX1	EX& BX2	PX	PE1	PE2	TY	LA1	LA2
Kirschsteiniothelia elaterascus	1.8	3.1	3.4	3.2	2.1	2.4	1.5	0.8	1.3	1.4	2.6	2.3	-	2.8	2.2	2.2	-
Lasiosphaeria ovina	1.6	1.3	3.0	1.6	2.6	1.8	1.1	1.8	1.0	1.5	1.1	1.8	-	1.2	1.2	1.0	-
Loculoascomycete sp. A242	1.6	2.0	2.9	1.7	1.7	1.6	1.4	0.6	1.1	2.9	1.3	2.1	2.1	1.4	1.3	1.3	-
Massarina sp. A25	2.4	3.4	5.2	2.7	2.4	1.4	1.0	1.9	3.2	1.0	1.2	2.2	-	1.3	1.2	1.4	-
Massarina ingoldiana	1.4	2.2	2.2	2.1	0.9	1.5	0.9	0.7	1.1	3.1	1.2	2.3	1.8	1.3	1.3	0.9	-
Pseudohalonectria sp. A251	1.5	1.2	1.8	1.6	1.5	1.4	1.0	1.5	1.3	0.9	1.2	1.6	-	1.3	1.3	0.8	1.9
Pseudoproboscispora caudae-suis	1.3	1.4	1.6	0.9	0.9	1.3	1.3	1.7	0.9	1.3	1.1	1.7	-	1.2	1.1	1.0	-
Submersisphaeria aquatica	1.1	0.7	1.1	0.9	1.0	0.8	0.9	1.0	0.8	0.6	1.1	1.0	-	1.1	1.0	0.8	1.4
Cercophora newfieldiana	1.8	1.2	2.2	1.8	1.2	2.2	1.1	0.9	1.4	1.0	0.8	2.1	1.5	1.0	1.2	1.2	-
Hymenoscyphus scutula	1.6	3.0	3.3	1.8	1.7	1.5	1.0	1.6	2.7	1.3	1.5	1.9	-	1.6	1.4	0.9	1.8
Loculoascomycete sp. A211	2.4	2.6	3.6	1.9	2.1	2.7	1.5	2.0	1.4	1.8	1.2	2.8	-	1.3	1.2	1.1	-
Ophioceras commune	2.8	3.5	3.1	3.0	3.8	3.0	2.7	1.9	1.4	3.1	2.5	3.7	-	1.6	-	1.4	-
Phomatospora sp. A5	1.3	1.3	2.6	1.3	1.3	1.1	2.4	1.6	1.5	1.0	1.1	1.2	-	1.1	1.1	1.0	-
Pyrenomycete sp. A3-1	2.7	1.6	1.4	1.5	2.1	1.6	1.2	1.2	0.6	1.4	2.6	2.1	-	2.9	3.9	2.4	-
Pyrenomycete sp. A54	1.4	1.3	2.5	1.0	1.6	1.2	0.9	3.2	3.3	1.4	0.9	1.4	1.6	0.9	0.8	0.7	-
Pyrenomycete sp. A-161	2.2	2.4	3.1	1.0	2.8	2.4	1.7	3.1	2.1	2.7	1.6	4.0	-	1.6	1.6	1.2	-

Table 3. Production of extracellular enzymes by freshwater ascomycetes*.

Species	LP	CL	ßG	EX&BX1	PX	PE1	PE2	TY	LA1	LA2
Ascovaginospora stellipala	++	+	-	++	+y	-	-	++	-	-
Chaetomastia typhicola	+	+	+	+	+w	-	+	++	++	-
Massariosphaeria scirpina	++	+	+	+	+w	+	+	+	+	-
Massariosphaeria sp. A-33	+	+	+	++	-	-	-	-	++	-
Ophioceras arcuatisporum	-	+	+	++	+y	-	-	++	++	-
Phaeosphaeria typharum	++	+	+	+	-	-	+	+	+	-
Pleospora sp. A168-1	++	+	+	++	-	-	-	-	-	-
Pyrenomycetes sp. A90-1	-	+	+	+	-	-	+	+	+	-
Annulatascus velatisporus	++	+	+	+	+w	-	-	-	+	-
Boerlagiomyces websteri	-	+	-	+	+y	-	+	+	+	-
Byssothecium flumineum	+	+	+	+	+w	-	+	+	++	-
Discomycete sp. A-93	+	+	+	+	+y	-	+	-	+	-
Halosarpheia heteroguttulata	++	+	+	+	+y	+	+	-	++	-
Jahnula sp. A322	++	+	+	++	-	-	+	+	+	+
Kirschsteiniothelia elaterascus	-	+	+	++	+y	-	-	-	++	-
Lasiosphaeria ovina	-	+	++	++	-	-	+	+	++	-
Loculoascomycete sp. A-242	++	+	+	+	+w	+	+	-	++	-
Massarina sp. A25	++	+	++	++	+y	-	+	+	++	-
Massarina ingoldiana	-	+	+	+	-	+	+	-	++	-
Pseudohalonectria sp. A251	-	+	+	+	+y	-	+	-	++	+
Pseudoproboscispora caudae-suis	++	+	+	+	+y	-	+	-	++	-
Submersisphaeria aquatica	+	+	++	++	+y	+	-	-	++	+
Cercophora newfieldiana	-	+	+	+	+y	-	-	++	+	-
Hymenoscyphus scutula	++	+	+	+	+y	-	+	+	++	+
Loculoascomycete sp. A211	-	+	+	+	+w	-	+	+	++	-
Ophioceras commune	-	+	+	++	+y	-	-	-	++	-
Phomatospora sp. A5	++	+	+	+	+w	-	+	-	+	-
Pyrenomycete sp. A3-1	-	+	+	++	+y	-	-	++	++	-
Pyrenomycete sp. A54	++	+	+	+	-	-	+	-	+	-
Pyrenomycete sp. A161	++	+	+	+	-	-	+	+	+	-

*LP: lipase; CL: cellulase; ßG: ß-glucosidase; EX&BX1: endoxylanase & ß-xylosidase (Pointing, 1999); PX: polyphenoloxidase; PE1: peroxidase (Pointing, 1999); PE2: peroxidase (Egger, 1986); TY: tyrosinase; LA1: laccase (Pointing, 1999); LA2: laccase (Pointing, 1999); ++ = strong reaction; + = weak reaction; - = no reaction; w = white colour; y = yellow colour.

Table 4. Production of extracellular enzymes by freshwater ascomycetes as measured by width of clearing or color reaction zone in cm*.

Species	AM	PR	PEC	PG	EN	EX&BX2
Ascovaginospora stellipala	1.2	-	0.3	0.2	0.2	0.5
Chaetomastia typhicola	0.7	1.4	0.9	1.2	0.2	0.5
Massariosphaeria scirpina	-	1.9	0.2	-	1.1	0.4
Massariosphaeria sp. A-33	0.7	2.4	-	0.5	0.1	0.1
Ophioceras arcuatisporum	0.1	-	-	-	-	1.6
Phaeosphaeria typharum	0.7	-	-	-	0.1	0.4
Pleospora sp. A168-1	-	-	0.6	0.1	0.2	0.8
Pyrenomycete sp. A90-1	0.5	-	0.2	-	0.7	0.9
Annulatascus velatisporus	0.6	3.1	-	-	0.3	1.1
Boerlagiomyces websteri	0.6	0.4	-	0.1	-	0.1
Byssothecium flumineum	0.8	0.3	-	-	0.1	0.8
Discomycete sp. A-93	1.5	2.4	-	-	-	0.7
Halosarpheia heteroguttulata	0.2	1.6	-	-	0.3	0.3
Jahnula sp. A322	0.5	1.2	0.6	0.2	0.6	0.4
Kirschsteiniothelia elaterascus	1.7	-	0.9	0.5	0.4	1.3
Lasiosphaeria ovina	0.8	-	-	-	0.2	0.3
Loculoascomycete sp. A-242	1.3	-	-	-	0.2	0.5
Massarina sp. A-25	0.5	1.6	1.0	1.8	0.2	0.1
Massarina ingoldiana	0.9	2.2	-	0.6	-	1.3
Pseudohalonectria sp. A251	-	-	-	-	0.5	0.1
Pseudoproboscispora caudae-suis	0.1	3.1	-	0.1	0.7	0.8
Submersisphaeria aquatica	0.2	0.9	0.3	0.1	0.4	0.8
Cercophora newfieldiana	1.2	1.6	-	-	0.1	0.5
Hymenoscyphus scutula	-	2.5	0.5	-	1.6	0.4
Loculoascomycete sp. 211	0.9	2.0	-	0.2	0.3	0.9
Ophioceras commune	0.2	-	0.5	0.2	0.3	0.3
Phomatospora sp. A5	1.2	2.6	0.2	-	0.3	0.9
Pyrenomycete sp. A3-1	0.5	-	0.2	0.1	0.3	0.1
Pyrenomycete sp. A54	0.6	1.8	0.4	-	0.3	0.5
Pyrenomycete sp. A161	-	1.6	1.1	-	0.3	0.1

*AM: amylase; PR: protease; PEC: pectinase; PG: polygalacturonase; EN: endoglucanase; EX&BX2: endoxylanase and ß-xylosidase (Pointing, 1999); - = no reaction.

and most of the other woody species gave weak pectinase reactions. In this study, 25% of the herbicolous species, 22% of the herbicolous/lignicolous and 50% of the lignicolous species were negative for both pectinase and polygalacturonase. Only four of 14 lignicolous species were positive for both pectinases and three of 14 lignicolous species were positive only for polygalacturonase.

Hemicelluloses are one of the three major components in plant cell wall. They comprise 25-40% of angiosperm woods and 25-30% of coniferous woods (Rayner and Boddy, 1988). The major hemicellulose of angiosperms is glucuromoxylan (15-30%), followed by glucomannan 2-3% (Pettersen, 1984). Hemicelluloses are the most easily hydrolyzed of the plant cell wall polysaccharide compounds. Xylans are the predominant compounds in the hemicellulose fraction (Flannigan, 1970). Xylanase has been shown to have applications for bleaching of wood pulp (Viikari *et al.*, 1986; Paice *et al.*, 1988; Senior *et al.*, 1991; Nakamur *et al.*, 1993). Gessner (1980) found that all the salt marsh isolates he tested exhibited xylanase activity. Zare-Maivan and Shearer (1988a) reported positive xylanase reactions for all lignicolous freshwater fungi tested. Marine ascomycetes also produce xylanase (Raghukumar *et al.*, 1994). In this study, two test procedures were used, xylan agar medium (Table 4) and RBB-Xylan agar medium (Table 3) (Pointing, 1999). All species of freshwater ascomycetes tested positive for endoxylanase and ß-xylosidase with both tests.

The most abundant polymer in wood, cellulose, may account for about 40-50% of dry weight of temperate woods (Pettersen, 1984). Native cellulose is partially crystalline and requires three hydrolytic enzymes acting synergistically for its complete degradation (Kirk and Cowling, 1984). All species in this study tested positive for cellulase. Endogluconase acts on crystalline and non-crystalline cellulose. In our study, all the species were positive for endogluconase except, *Boerlagiomyces websteri*, Discomycete sp. A-93, *Massarina ingoldiana*, and *Ophioceras arcuatisporum* (Table 4). Cellulase activity has been recorded for other aquatic fungi, including salt marsh fungi (Gessner, 1980), aquatic hyphomcetes (Suberkropp *et al.*, 1983), and freshwater lignicolous fungi (Zare-Maivan and Shearer, 1988a,b). Rohrmann and Molitoris (1992) recorded cellulase activity on acid-swollen avicel for marine ascomycetes and Raghukumar *et al.* (1994) recorded about 80% of the marine species they tested were positive for cellulotic enzymes. Highley (1980) reported the inability of extracellular enzyme preparations from several species of brown-rot fungi grown on avicel to release reducing sugars from a similar substrate in vitro, concluding that activity was lacking (Reese *et al.*, 1950). Montencourt and Eveleigh (1977) working with the highly

cellulolytic *Trichoderma reesi*, found that this fungus failed to produce 'clearing zones' in acid swollen cellulose plates unless the surfactant photon D was added. They suggested that this compound may promote the release of cell bound enzymes to permit increased digestion of cellulose.

Lignins are complex aromatic polymers formed from phenyl propenoid subunits (Kirk and Obst, 1988). They comprise 20-30% of woody tissue (Pettersen, 1984) and form a physical barrier that protects cellulose and hemicellulose from degradative enzymes. Cellulose microfibriles are coated or overlaid by hemicelluloses, which are covered by a lignin sheath (Kirk and Cowling, 1984). Laccase enzymes are commonly produced by wood-decay fungi (Reinhammar, 1984; Leonowicz *et al*., 1985; Mayer, 1987) and has been implicated in fungal degradation of lignin and its derivatives (Galliano *et al*., 1988; Archibald and Roy, 1992; Higuchi, 1993). Laccase has been reported to depolymerise lignins and various lignin model compounds (Galliano *et al*., 1988; Archibald and Roy, 1992; Bourbonnais and Paice, 1992; Higuchi, 1993). Laccase plate assays are easy, quick, and convenient (Sundman and Nase, 1971; Niku-Paavola *et al*., 1990). They are based on the ability of an organism to oxidize compounds such as ABTS (2,2-azino-bis-[3-ethylbenzene-6-sulfonic acid]), guaiacol, and syringaldazine incorporated in the media resulting in intense color production next to the fungal colony. Laccase has been correlated with white rot decay (Ander and Eriksson, 1976). Platt *et al*. (1984) suggested that preliminary activity by laccase is required for lignin degradation.

In the present study, two different methods were used to detect laccase activity (Pointing, 1999). The first was the ABTS agar method. For this method, colorless agar medium turns green due to the oxidation of ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) to ABTS- azine in the presence of laccase (Nika-Paavola *et al*., 1990). Only two species, *Ascovaginospora stellipala* and *Pleospora* sp. A-168 (species from herbaceous substrates) had negative laccase activity using the first method (Table 3). The second method was the well test of syringaldazine (4-hydroxy-3, 5-dimethoxybenzaldehyde) (Pointing, 1999). All species were negative for laccase activity using the second method, except for, *Hymenoscyphus scutula*, *Submersisphaeria aquatica* and *Jahnula* sp. A-322, (Table 3). Zare-Mavin and Shearer (1988a) showed that of 18 species of freshwater lignicolous fungi, 66% produced laccase on malt extract medium and 33% on cornmeal agar. Clearly the method and/or medium used influences the results of laccase plate tests. Raghukumar *et al*. (1994) found that the laccase plate assay was much less reliable than spectrophotometric laccase determination using extracellular culture fluid from fungal culture grown in liquid media. They found that several identified and unidentified taxa of marine fungi isolated from the coast

of India from decaying mangroves and sea grass tested positive for the presence of laccase (Raghukumar *et al.*, 1994). Bourbonnais *et al.* (1997) recorded that laccase also generates radicals from a low-molecular-mass redox mediator, but in an H2O2- independent reaction. The mediator compound has been identified as 3-hydroxyanthranilate in the laccase producing white-rot fungus *Pycnoporus cinnabarinus*. Although several artificial mediator compounds such as 1-hydroxybenztriazole (HBT) and 2,2`-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) are also capable of acting as laccase mediators (Pointing *et al.*, 2000).

In this study, five species were positive for peroxidase activity using the method of Pointing (1999) while 20 species produce peroxidase using the method of Egger (1986). In the presence of H2O2, peroxidase catalyze oxidation of an endogenously generated low-molecular mass redox mediator veratryl alcohol (Reddy and D' Souza, 1994), which in turn carries out an one-electron oxidation of non-phenolic aromatic nuclei in lignin to generate aryl cation radicals. These then degrade non-enzymatically to aromatic and aliphatic products, which are mineralized intercellularly. The radicals generated can carry out a variety of reaction, including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization / polymerization, and demethylation (Pointing, 2001).

Tyrosinase, a common enzyme acting in melanin biosynthesis, is assumed to participate also in lignin degradation by the detoxification of its breakdown products. Lyr (1958) found tyrosinase for 62 out of 154 species of wood-decaying fungi. Gessner (1980) recorded about half of the salt marsh fungi he tested were positive for tyrosinase activity when grown on a malt extract medium. In our study, approximately 50% of the species were positive for tryosinase. Zare-Maivan and Shearer (1988a) showed that tyrosinase production depends on the medium used, since on malt extract agar only 10% of the strains of freshwater lignicolous fungi produced this enzyme, whereas on cornmeal agar, tyrosinase was positive in 45% of the strains.

In our study, 70% of the species produced phenoloxidizing enzymes. Most of the herbaceous species were negative for phenoloxidases enzymes (Table 3). The physiology of Lip (peroxidase), MnP (polyphenoloxidase) and Lac (laccase) has been extensively studied using submerged liquid cultures and is comprehensively reviewed elsewhere (Buswell and Odier, 1987; Boominathan and Reddy, 1992; Wariishi *et al.*, 1992; Hattaka, 1994; Reddy and D' Souza, 1994; Thurston, 1994; Orth and Tien, 1995; Leonowicz *et al.*, 1999). They are often referred to as lignin-modifying enzymes or LME (Pointing, 2001). LME production occurs during secondary metabolism and is induced by limited nutrient levels, particularly nitrogen. Some taxa have,

however, been demonstrated to produce Lip, MnP and Lac under conditions of nitrogen sufficiency (Buswell *et al.*, 1984). Production of Lip and MnP is generally optimal at high oxygen tensions, but is repressed by agitation of fungi grown in submerged liquid culture. Genes encoding LiP, MnP (Gold and Alic, 1993; Reddy and D' Souza, 1994), and Lac (Mansur *et al.*, 1997) have been characterized, and current evidence suggests that all three enzymes are encoded by gene families that allow complex regulation, and production of multiple isoforms. Recent research has shown that nutrient nitrogen levels, mediator compounds, and required-metal (i.e. Mn^{2+} for MnP, Cu^{2+} for Lac) concentrations affect transcription levels of LiP (Li *et al.*, 1994), MnP (Ruiz-Duenas *et al.*, 1999), and Lac (Collins and Dobson, 1997; Palmieri *et al.*, 2000).

This study demonstrates that freshwater ascomycetes are able to produce many extracellular enzymes important in the decomposition of plant structural materials. It appears that there may be some specialization in the types of enzymes produced between lignicolous and herbicolous species. Further work using natural substrates and additional taxa is needed to better understand the role of freshwater ascomycetes in decomposing submerged plant substrates and species specificities in types of substrates decomposed.

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