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Amazonian Lignocellulosic Materials-I

Fungal Screening from Decayed Laurel and Cedar Trees

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ABSTRACT

Fungi were screened for important industrial enzymes produced from industrial chips and sawdust of laurel (*Louro inamui, Ocotea cymbarum*) and cedar (*Cedro, Cedrella odorata*). Seven hyphomycetes and one zygomycete were isolated and characterized. Two different media for testing the enzymatic activities were used. In general, in potato dextrose (1%) medium (M-3) a preponderancy of ligninolytic over cellulolytic enzymes was observed. In potato infusion-sawdust wood (1%) medium (M-4) the cellulolytic, xylanolytic, and lipolytic enzymes were efficiently induced. Significant modulation of enzyme production by the carbon source was found in the extracted fungi from self-heated industrial chips piles of cedar and laurel trees at the Amazonian region.

Index Entries: Amazonian wood; thermophilic; cedar and laurel trees, ligninases; cellulases; xylanases; lipase; lactase; amylase.

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INTRODUCTION

Thermophilic fungi are those species that have a maximum temperature for growth at or above 50°C, and a minimum temperature for growth at or above 20°C; thermotolerant fungi share this ability to grow at 50°C, but also grow below 20°C (*1*). The thermophilic species are most frequently found in self-heating environments (*2,3*) such as industrial chip piles or sawdust (e.g., Sweden [*4*]; Canada [*5*]; USA [*6,7*]; and in Brazil [*8*]).

The thermophiles grow on chips and sawdust, and their ability to degrade cellulose confirmed the destructive role of these fungi on woods. However, the fungi could be useful, especially in the pulp and paper industry (*1*) since they also produce several other enzymes of industrial interest; for example, Aspergillus and Trychoderma produced glucoamylases, cellulases, and proteases, among others (*9*). The world market for the enzyme industry is worth $625 million (*10*). About 62% of the total enzymes produced are applied to food, 33% to detergents, and 5% to the textile industry. From all of these enzymes 60% are proteases, 30% are carbohydrases, 3% are lipases, and the remaining 7% are special enzymes (*11,12*). The most important areas in the food industry are starch, cheese, juices, and bread production (*13*). Recently, food industry applications of enzymes involving amylases, proteases, and lipases have been reviewed (*14*). Lactase application was also recently discussed (*15*).

There has been an increased interest for xylanases (*16*), cellulases (*17,18*), and ligninases (*19*) in the pulp and paper industry. In the Kraft effluent treatment a great interest in using ligninolytic fungi and enzymes are now in progress (*20–23*) as substitutions or complements of classic treatments.

The present study documents the occurrence of these fungi in self-heating wood chip piles of laurel and cedar trees in Manaus (in the Amazon region), and evaluates the industrial potential of these different kind of enzymes.

MATERIALS AND METHODS

Collection of Fungi

The fungi were collected at the CR/12 Section of Military Regional Commission at Manaus, Amazonian State, Brazil, following perfusion technique as previously described (*24,25*).

Chips and Sawdust Piles

The fungi were collected from industrial chips and sawdust of a laurel tree (*Louro inamui, Ocotea cymbarum*) and a cedar tree (Cedro, *Cedrella odorata*) that were in decomposition for 10 yr under high humidity and sun at Manaus, Amazonian State, Brazil.

Culture Medium

Collection

The following medium was used for the collection: Agar-Sabouraud, agar-malt-yeast extract (1%), and potato dextrose-agar.

Preservation

For the Fusarium, Trichoderma, and Gliocladium genera, potato dextrose-agar was used. For Geotrichum and Mucor genera, Sabouraud media was used. For Penicillium and Aspergillus genera, Agar-Czapek was used.

Enzyme Production

For all the genera under study the preinoculation was prepared in two different conditions: Potato dextrose-agar with sawdust wood (around 200 mg) on the surface of the solidified medium (M-1); and potato infusion-agar with the sawdust wood (around 200 mg) (M-2). The mycellium and spore growing in this medium was inoculated in a liquid culture of potatoes dextrose (1%) (M-3); and in the other sets potato infusion-sawdust wood (1%) (M-4).

Chemical Analyses of the Organic Material Where the Fungi were Collected

Chip and sawdust piles were analyzed by standard methods, as described previously (*19*). The initial components were the following: $2.0 \pm 0.3\%$ extractives; $37.1 \pm 0.8\%$ Klason lignin; $1.2 \pm 0.1\%$ soluble lignin; $32.6 \pm 0.2\%$ cellulose, and $9.8 \pm 0.8\%$ hemicellulose.

Fungi Characterization

Aspergillus and Penicillium were identified by Maria J. dos Santos Fernandes, and Hyphomycetes and Coelomycetes by Maria A. de Queiroz Cavalcante from Mycology Department, Centro de Ciencias Biológicas da Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. The methodology described by Attili (*26*) and the specialized literature in this area (*27–31*) was followed. All the collected strains were maintained in mineral oil, silica gel and agar/water blocks (*32*).

Enzymatic Activities

Cellulases (*33*)

0.5 mL of culture filtrate and 1 mL of citrate buffer 0.05*M*, pH 4.8, were added to a test tube containing Whatman No. 1 filter paper (rolled in a 1×6 cm strip). After 1 h of incubation at 50°C, the reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNS) method. The DNS method (*34*) consisted of the addition of 1,5 mL of sample and 3 mL of DNS reagent and incubation at 100°C for 5 min. After cooling, the absorbance

at 550 nm were measured and compared with a standard curve with glucose as substrate. DNS reagents consisted in 10.6 g of 3,5-dinitrosalicylic acid, 19.8 g NaOH, 306 g of sodium potassium tartrate, 7.6 mL phenol, and 8.3 g sodium bisulfite.

Endo-1,4-Beta-D-Glucanase (33)

0.5 mL of the culture filtrate was added to 0.5 mL of a 1% carboxymethylcellulose (CMC) in citrate buffer 0.5*M*, pH 4.8. The mixture was incubated at 50°C for 30 min and the reducing sugars were measured by DNS method.

Beta-Glucosidase and Beta-Xylosidase (35)

This method used *p*-nitrophenyl-beta-D-glucopyranoside (pNPG) as substrate for beta-glucosidase, and *p*-nitrophenyl-beta-D-xylopyranoside (pNPX) as substrate for the beta-xylosidase activities. To 0.1 mL of culture filtrate, 0.4 mL of 0.2% (w/v) of pNPG or pNPX, in 50 m*M* acetate buffer, pH 4.8, were added, and incubated at 50°C for 30 min. After incubation, the reaction was interrupted by addition of 1 mL sodium bicarbonate 10%. The *p*-Nitrophenol produced was measured at 410 nm and compared with a standard curve with *p*-nitrophenol.

Xylanase Activity (36)

To 0.5 mL of culture filtrate, 0.5 mL of a 1% xylan in acetate buffer 50 m*M*, pH 5.0, was added. After incubation for 30 min at 50°C the reducing sugars were measured by the DNS method using xylose as standard.

Lignin Peroxidase (37)

To 550 μL of culture filtrate, 200 μL of H_2O_2 2 m*M*, and 250 μL of a veratryl alcohol solution of 8 m*M* in sodium tartrate buffer 0.4*M*, pH 3.0, were added. Veratryl aldehyde was determined at 310 nm ($E = 9.300\ M^{-1}\ cm^{-1}$).

Phenoloxidase Activity (38)

1. *Syringaldazine Method:* Laccase activity ws measured spectrophotometrically with syringaldazine at 525 nm ($E = 65{,}000\ M^{-1}\ cm^{-1}$). To 0.5 mL of culture filtrate and 0.4475 mL of 0.1*M* tartrate buffer, pH 4.2, 25 μL of syringaldazine (stock solution 5 mg/10 mL ethanol) and 0.5 mL of water was added. Peroxidase activity was measured following the same procedure except that 0.5 mL of water was substituted by 0.5 mL of H_2O_2 (Stock solution 2 m*M*).
2. *o-Dianisidine Method:* Laccase activity was measured with o-dianisidine at 460 nm ($E = 29{,}400\ M^{-1}\ cm^{-1}$) (modified by Eriksson et al. [*39,40*]) at the pH of the culture filtrate. To 1 mL of 1.0 m*M* o-dianisidine, 1 mL of culture filtrate and 1 mL of water was added, and the absorption was measured after 5 min. The peroxidase activity was carried out using the same

method as before, except that 1 mL of water was replaced by 1 mL of 2 m*M* H_2O_2.

Amylase Activity (17,41)

1 g of soluble starch in 100 mL of 0.02*M* sodium phosphate buffer, pH 6.9, was dissolved. The insoluble part of this solution was eliminated. To 1 mL of properly diluted culture filtrate, 1 mL of stock solution of soluble starch was added and incubated for 3 min at 20°C. The reducing sugars were measured by DNS reagents as previously described. Maltose was used as the standard curve (0.2–2.0 mg in 2 mL of water). The unit was the mg of maltose/3 min by 1 mL of culture filtrate.

Lipase-Esterase (42)

The reaction was initiated by adding 0.5 mL of culture filtrate to a 3 mL cuvette containing 0.1 mL (100 μg) of *p*-nitrophenyl acetate (stock solution 1 mg/mL methanol) and 2.4 mL of a 0.06*M* sodium phosphate, pH 7.0. The increase in optical density at 400 nm during the incubation at room temperature was measured. One unit is the increase of 0.01 absorption units at 400 nm/min by mL.

Lactase (Galactosidase) (43,44)

To 0.1 mL of a 1*M* NaCl, 0.5 mL of *o*-nitrophenyl-beta-D-galactopyranoside (ONPG) (stock solution 50 mg/10 mL 0.05*M* Tris Buffer, pH 7.6) was added into a cuvette, and the reaction was started by addition of 0.4 mL culture filtrate, incubated at 25°C, and the absorption was measured at 405 nm. One unit is the change in 0.01 OD/min by mL.

Protease (45)

A stock solution of the substrate containing 25 mg of azocasein and 5 mg of sodium bicarbonate per mL (prepared dissolving 250 mg azocasein in 5 mL of 1% $NaHCO_3$ at 60°C with stirring, the pH adjusted to 8.3, and the solution diluted to 10 mL with distilled water). The stock solution was stored at 0°C. The substrate solution was maintained at 38°C and 1-mL aliquot of the stock solution was mixed with 1 mL of culture filtrate. After 30 min incubation was stopped by addition of 8 mL of 5% trichloroacetic acid, including the blank. The content of each tube was centrifuged for 8 min at 7000 rpm. To a 1-mL aliquot of the filtrate, 1 mL of 0.5*N* NaOH was added and the color was read at 440 nm. One unit is the amount of enzyme that catalyzes the release of azo dye by changing 0.001 OD/min.

RESULTS AND DISCUSSION

From the first screening (58 strains) we have selected a representative species of each genera for a detailed study, related with their growth and enzymatic production.

We have isolated and characterized seven hyphomycetes (*Trichoderma pseudokoningii* [185] mesophilic, *Gliocladium virens* [220] noncharacterized, *Trichoderma harzianum* [264] mesophilic, *Penicillium citrinum* [267] thermophilic, *Fusarium oxysporum* [282] noncharacterized, *Aspergillus flavus* [288] mesophilic, and *Geotrychum candidum* [293] thermophilic-mesophilic); and 1 zygomycete (*Mucor sp.* [262] thermophilic) (*46*).

In the isolation process a universal culture medium was selected where all fungi grew adequately (e.g., Agar, Sabouraud, Potato dextroxe-agar, and Agar-malt extract [1%]). For preservation, a specific medium for the different genera was used for identification and taxonomic characterization in order to maintain the important morphological characteristics. For the endogenous stimulation, in some cases, a certain amount of the substrate source of the natural fungal growing, for example, as sawdust wood that was in the self-heated wood piles, was added to the culture medium. This strategy was followed to conserve the morphological characteristics of the fungi at a macroscopic level.

In the enzymatic production, two types of liquid culture medium were tested, one with a glucose source and the other in its absence, in order to test the best condition for the fungi. It was observed that the activities of the detected enzymes were culture dependent. (Table 1 and Table 2).

Table 1 shows the enzymatic activities in culture medium M-3. In this medium six of the total of eight were good ligninolytic fungi. None of these fungi produced phenoloxidase as tested by syringaldazine or *o*-dianisidine methodology. Interesting is the case of *T. harzianum* [264] in which a similar lignin peroxidase activity as in *Chrysonilia sitophila* (*47,48*) was found, but no cellulase activity, presence of beta-glucosidase, or xylanase activities, indicating an excellent fungi for pulp biobleaching and Kraft effluent treatment (*21*). In general, amylases and xylanases are not strongly affected by the different culture media.

From Table 2 it is possible to conclude that lignin peroxidase and protease were not affected by different culture medium, but notoriously the cellulase activities were increased. Lipase activity was strongly influenced by the presence of sawdust.

In summary, enzyme production is modulated by a carbon source, and the fungus *T. harzianum* [264] appeared as a potential source in effluent treatment, and also with a good potential in lipase-esterase activity. Presumably, the self-heated industrial chip piles are excellent places for fungal screening for lignocellulosic activities.

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Table 1
Enzymatic Activities of the Selected Fungi Strains[a]

Fungi	Lignin peroxidase, U/L	Phenoloxidase, U/L	Filter paper activity, U/L×10^{-3}	Beta-glucosidase, U/L×10^{-3}	Endo-1,4-beta-D-glucanase, U/L×10^{-3}	Xylanase, U/L×10^{-3}	Beta-xylosidase, U/L×10^{-3}	Protease, 0.001 OD/min mL	Lactase, 0.01 OD/min mL	Amylase, mg/mL	Lipase, 0.01 OD/min mL
[185]	12	0	10	1.0	0.7	28	0.8	ND[b]	20.2	1.1	5.3
[220]	0	0	23	ND	ND	51	ND	ND	ND	ND	ND
[262]	0	0	4	ND	ND	21	ND	ND	ND	ND	ND
[264]	23	0	0	1.4	0	18	1.0	ND	23.3	1.3	8.8
[267]	24	0	4	ND	ND	27	ND	ND	17.7	1.6	9.1
[282]	13	0	4	2.5	1.5	32	5.4	0.5	14.5	1.5	8.9
[288]	17	0	8	ND	ND	26	ND	ND	ND	ND	ND
[293]	13	0	0	ND	ND	ND	ND	ND	ND	ND	ND

[a] Culture Medium M-3.
[b] ND = not determined.

Table 2
Enzymatic Activities of the Selected Fungi Strains[a]

Fungi	Lignin peroxidase, U/L	Phenoloxidase, U/L	Filter paper activity, U/L×10^{-3}	Beta-glucosidase, U/L×10^{-3}	Endo-1,4-beta-D-glucanase, U/L×10^{-3}	Xylanase, U/L×10^{-3}	Beta-xylosidase, U/L×10^{-3}	Protease, 0.001 OD/min mL	Lactase, 0.01 OD/min mL	Amylase, mg/mL	Lipase, 0.01 OD/min mL
[185]	ND[b]	0	5	ND	ND	24	ND	1.1	44.6	1.3	20.6
[220]	ND	0	16	1.9	20	43	0.6	0.5	13.6	1.0	12.3
[262]	11	0	7	1.4	0	29	1.4	1.1	ND	1.4	18.9
[264]	19	0	13	ND	ND	49	ND	ND	46.6	1.3	24.1
[267]	19	0	65	ND	ND	52	ND	0.9	29.8	ND	17.7
[282]	ND	0	0	ND	ND	29	ND	ND	ND	ND	14.1
[288]	ND	0	0	1.3	0	69	1.1	0.6	ND	1.0	24.3
[293]	ND	0	0	ND	ND	24	ND	ND	ND	ND	ND

[a] Culture Medium M-4.
[b] ND = not determined.

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