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Enzymes Produced by Soil Fungi Following Microaerobic Growth on Lignocellulosic Materials

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Abstract

Four fungal strains able to grow under low oxygenation conditions were selected and used in studies to determine the production of enzymes (endoglucanases, exoglucases, β-glucosidase, and peroxidases) that promote the degradation of lignocellulosic materials. The capacity of the fungi to ferment lignocellulosic materials was also investigated. Avicel, xylan, Whatman no. 1 filter paper, or agroindustrial residues were used as carbon sources in a medium containing mineral salts, vitamins, and cysteine as a reducing agent, under either microaerophilic or combined conditions (aerobic followed by microaerophilic conditions). The results obtained with strains Q10, H2, and LH5 suggest that they prefer a low oxygen concentration for growth and enzyme production. However, strain F20 seems to need higher levels of oxygenation. Lignocellulolytic activities were detected in all strains but varied with the carbon source used for growth. In general, the highest levels of these activities were produced by strain H2 under microaerophilic conditions. Ethanol and other nongaseous fermentation products were detected following high-performance liquid chromatography analysis using a Supelcogel C-610H column, demonstrating the fermentative capability of these strains.

Index Entries: Cellulolytic fungi; lignocellulases; lignocellulose degradation; xylanases.

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Introduction

Lignocellulosic biomass is a unique renewable resource. The major fraction of this material, typically 35–50%, is cellulose, a polymer of glucose. Cellulose is the most abundant organic compound on Earth and is the main constituent of the cell walls of plants. Hemicellulose, the next largest fraction, is also a polymer of sugars, whose types and distributions vary depending on the particular biomass source. For many types of lignocellulosic material, xylan, a polymer of xylose, represents the predominant fraction of the hemicellulose component. The third largest fraction, of about 15–25%, is typically lignin, a phenylpropane polymer of complex composition *(1)*. Together, cellulose and hemicellulose compose about 65–75% of the overall lignocellulosic biomass, which could be broken down to their component sugars, making them available for fermentation *(2)*.

Microbial degradation of cellulosic biomass not only represents an important part of the carbon cycle within the biosphere, but also has attracted the attention of biotechnologists to the use of microbial enzymes in the treatment of cellulose for the generation of fermentable sugars *(3,4)*.

An association of enzymes acting in a concerted fashion degrades cellulose. This cellulase enzyme system consists of three major components, which are often referred to as endoglucanase (carboxymethylcellulase [CMCase]), exoglucanase (avicelase), and cellobiase (β-glucosidase) *(5–7)*. These enzymatic components act synergistically in the hydrolysis of crystalline cellulose *(8)*.

Cellulolytic microorganisms are found among various taxonomic groups. They include fungi and bacteria, aerobes and anaerobes, and mesophiles and thermophiles, and occupy a variety of habitats. Hemicellulose is subject to enzymatic degradation by a wide variety of fungi and bacteria. Most cellulolytic organisms are usually also xylanolytic. When acting on hemicellulose, many xylanolytic microorganisms produce more than one form of enzyme *(9)*.

Only a few groups of microorganisms are capable of degrading the complex lignin polymers, and they are best exemplified by the white-rot fungi, which cause the greatest degree of mineralization. Lignin is degraded during secondary metabolism, which is triggered by limitation of nitrogen, carbon, or sulfur *(10,11)*. The process by which these fungi degrade lignin is oxidative, involving enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and H_2O_2-producing oxidase *(12)*.

Most of the lignocellulose in nature is oxidized to carbon dioxide by aerobic microorganisms, but a substantial amount is degraded in anaerobic environments, such as soil and mud, that contain plant material. Lignin appears relatively inert and recalcitrant to microbial decomposition in a variety of neutral to acidic anoxic environments *(13,14)*. Oxygen concentrations affect lignin degradation, and the greatest ligninolytic activities have been associated with high oxygen concentrations *(15)*. Oxygen concentrations within tree trunks, however, are extremely low *(16)*, and decompos-

ing logs in a rain forest would not be expected to have an environment of high oxygen. Since ligninolytic fungi growing in these logs do not apparently possess any unique system to tolerate low oxygen concentrations, some mechanism that delivers the oxygen necessary for the reactions involved in lignin degradation must be operative *(15)*.

The microbial production of chemicals by lignocellulose fermentation has generated considerable research interest *(12)*. Since the commercially available celluloses are unlikely to be used as substrates for industrial fermentations, fuel production from biomass in the form of agricultural and forest residues is attractive as an alternative renewable energy resource *(2,17,18)*.

The aim of this study was to determine the lignocellulolytic activities and the major nongaseous fermentation compounds produced following the growth of four fungal strains under microaerophilic and combined (aerobic followed by microaerophilic) conditions, in media containing various lignocellulosic materials as the carbon source.

Materials and Methods

Microorganisms

Four strains of cellulolytic fungi were used in this study. Strains Q10 (*Trichocladium canadense*, ATCC 201360) and H2 (basidiomycete specie) were isolated under anaerobic conditions as described by Durrant et al. *(19)*. Strains F20 and LH5 were isolated from soil samples collected around Campinas, Sao Paulo, Brazil. Preliminary microscopic observations of these two strains indicate that they belong to the genus *Aspergillus*.

Media and Culture Conditions

The four fungal strains were cultivated in various carbon sources, such as filter paper (Whatman no. 1), Avicel, xylan, sawdust, and sugar cane bagasse, in a defined liquid medium containing 1.0 g/L of KH_2PO_4, 0.54 g/L of NH_4Cl, 0.3 g/L of $CaCl_2$, 1.0 g/L of urea, 1.0 g/L of L-cysteine hydrochloride, and 1.0 mL of resazurin solution (0.1% [w/v]); 1.0 mL of a mineral solution (0.22 g/L of ammonium tartrate, 0.66 g/L of manganese sulfate, 0.15 g/L of iron sulfate, 0.10 g/L of cobalt sulfate, 0.10 g/L of zinc sulfate, 6.4 mg/L of copper sulfate, and 10 mg/L of aluminum potassium sulfate); and 0.5 mL of a vitamin solution (2.0 mg/L of biotin, 2.0 mg/L of folic acid, 5.0 mg/L of thiamine-HCl, 5.0 mg/L of riboflavin, 10.0 mg/L of pyridoxine-HCl, 0.10 mg/L of cyanocobalamine, 5.0 mg/L of nicotinic acid, 5.0 mg/L of DL-calcium panthothenate, and 5.0 mg/L of thiotic acid), at pH 5.0. The final concentration of the carbon sources was 0.5% (dry wt/v).

Inoculated Erlenmeyer flasks (50 mL of medium/100-mL flask) were incubated under both microaerophilic and combined conditions. Inoculated Erlenmeyer flasks containing filter paper medium were also incubated under both stationary and shaken (150 rpm) conditions. Microaerophilic conditions were achieved by using sealed jars containing Microaerobac

plates (Probac, São Paulo, Brazil). These plates generate a reduced oxygen (5–15% O_2), enriched carbon dioxide (10% CO_2) environment within the incubation jars. Through oxidation-reduction reactions, hydrogen and carbon dioxide are generated following the addition of water to the plates. The hydrogen formed combines with the oxygen present in the closed jars, forming water, which condenses on the walls of the jars. To ensure good conditions for the cultivation of microaerophilic microorganisms, such as *Campylobacter* and *Helicobacter*, the system was activated and incubated according to the manufacturer's instructions. The low oxygenation was guaranteed by observing the disappearance of the resazurin's blue color in uninoculated control flasks. The blue color was reestablished when the flasks were in contact with oxygen, following the opening of the jars. Supernatant samples were collected following 20 and 35 d of cultivation. For the combined conditions, inoculated flasks were first incubated under shaking at 150 rpm for 6 d followed by incubation under microaerophilic conditions, giving a total growth period of 20 d. The supernatant fluids were collected via filtration and used for the determination of enzyme activities and fermentation products.

Enzyme Preparation and Assays

CMCase, avicelase, and xylanase activities were determined by measuring the production of reducing sugars from either carboxymethylcellulose (sodium salt, low viscosity; Sigma, St. Louis, MO), Avicel, or xylan (oat spelt; Sigma), by means of the dinitrosalicylic acid method *(20)*. β-Glucosidase activity was assayed using *p*-nitrophenyl-β-D-glucanopyranoside as the substrate. The *p*-nitrophenol produced was measured spectrophotometrically at 410 nm after the addition of 2.0 mL of 1 *M* $NaCO_3$. All enzymatic reactions were carried out for 30 min at 50°C. Enzyme activities were expressed as international units per liter.

The ligninolytic activities were determined spectrophotometrically and the results were expressed in international units per liter. LiP and veratryl alcohol oxidase were assayed by the method of Tien and Kirk *(21)* with veratryl alcohol as a substrate. MnP was assayed by measuring oxidation of phenol red *(22)*. Laccase and peroxidases were determined with syringaldazine as the substrate *(23)*.

Determination of Soluble Fermentation Products

The culture filtrates were assayed by high-performance liquid chromatography (HPLC) to determine the presence of any fermentation products. An HPLC system (Shimadzu, Tokyo, Japan) model LC-6A fitted with an ultraviolet (UV) detector model SPD-6A and a CR-4A integrator was used. A Supelcogel C610H (30 cm × 7.8 mm) column and 0.1% H_3PO_4 as the mobile phase were used. The fermentation products were detected at 210 nm. When using UV detection, the peak areas of the chromatograms are not consistently related to concentrations, even though the retention

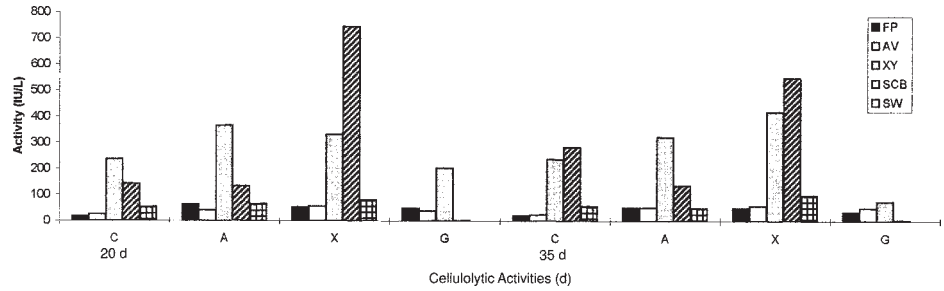

Fig. 1. Cellulolytic activities during growth of *T. canadense* (Q10) under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing filter paper (FP), Avicel (AV), xylan (XY), sugar cane bagasse (SCB), or sawdust (SW) as the carbon sources. Enzyme activities: CMCase (C), avicelase (A), xylanase (X), and β-glucosidase (G).

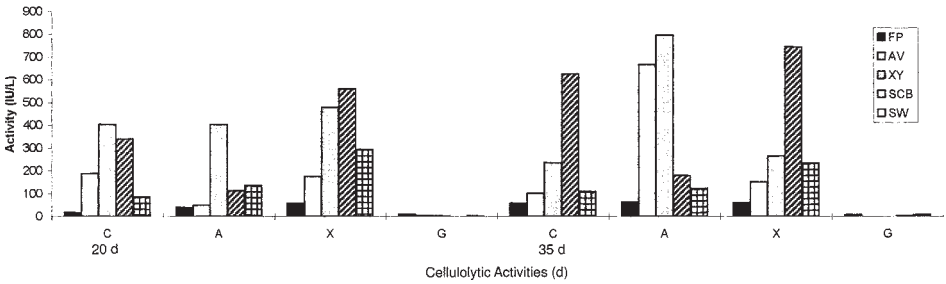

Fig. 2. Cellulolytic activities during growth of strain H2 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing filter paper (FP), Avicel (AV), xylan (XY), sugar cane bagasse (SCB), or sawdust (SW) as the carbon sources. Enzyme activities: CMCase (C), avicelase (A), xylanase (X), and β-glucosidase (G).

times do allow identification of the sample components. We are currently investigating the use of refractive index detection for quantitative analysis.

Substrate Utilization

The ability of the isolates to utilize various soluble compounds as substrates for growth was determined by estimating visually the increase in mycelial development after three transfers in liquid media containing the potential substrate. The final substrate concentration was 0.5% (w/v). Growth was assessed as good, moderate, or weak *(19)*.

Results and Discussion

Determination of Enzyme Activities

As shown in Figs. 1–4, the four fungi grew and produced cellulolytic enzymes under microaerophilic conditions, regardless of the time of cultivation. Growth in xylan and sugar cane bagasse resulted in the production of the highest levels of cellulases and xylanases by the four strains.

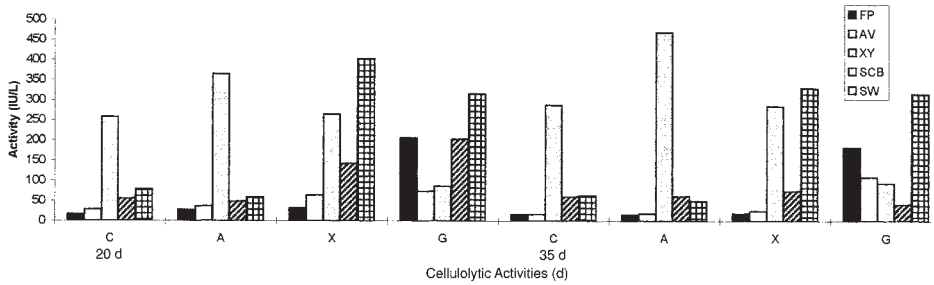

Fig. 3. Cellulolytic activities during growth of strain LH5 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing filter paper (FP), Avicel (AV), xylan (XY), sugar cane bagasse (SCB), or sawdust (SW) as the carbon sources. Enzyme activities: CMCase (C), avicelase (A), xylanase (X), and β-glucosidase (G).

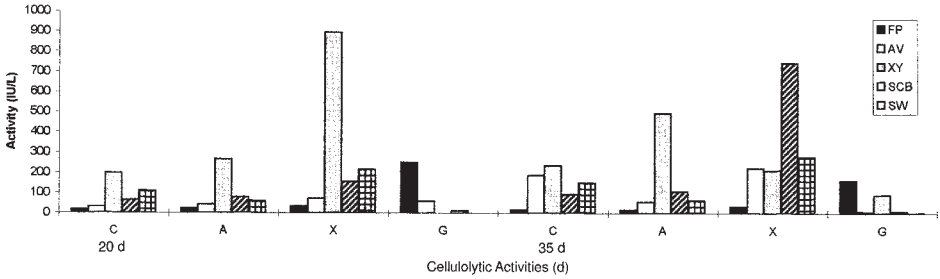

Fig. 4. Cellulolytic activities during growth of strain F20 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing filter paper (FP), Avicel (AV), xylan (XY), sugar cane bagasse (SCB), or sawdust (SW) as the carbon sources. Enzyme activities: CMCase (C), avicelase (A), xylanase (X), and β-glucosidase (G).

T. canadense (Q10) produced the best activities following its growth in medium containing xylan or sugar cane bagasse as the carbon source (Fig. 1). Considering the production of cellulases and xylanases, the best growth substrate for Q10 was xylan. Strain H2 (Fig. 2) showed an increase in avicelase activity after 20 d of incubation, which was much higher in Avicel or xylan as the carbon sources. No β-glucosidase activity was produced by this strain. As shown in Fig. 3, with the exception of β-glucosidase, xylan was the best substrate for the production of cellulolytic enzymes by strain LH5. Strain LH5 was the best producer of β-glucosidase, and the highest activities were obtained following its growth in sawdust, filter paper, or sugar cane bagasse. In general, strain F20 (Fig. 4) showed the lowest level of the cellulolytic activities among the four strains, with the exception of xylanase produced in xylan (20 d). However, strain F20 showed the highest level of cellulolytic activities following its growth under combined conditions (Fig. 5), suggesting that the aeration provided during shaking for 6 d before changing it to microaerophilic growth may have been an important factor for this strain.

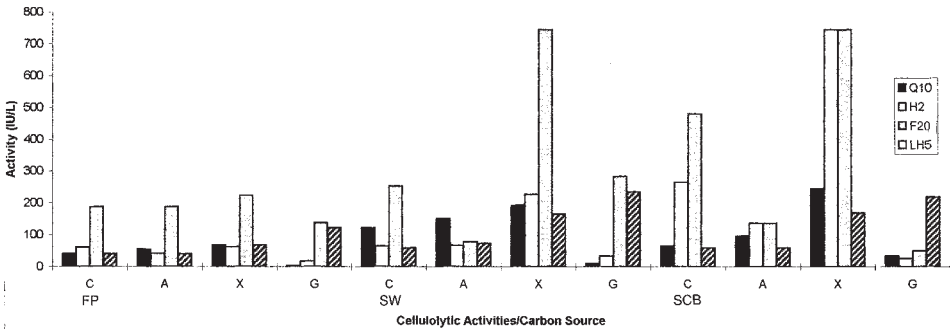

Fig. 5. Cellulolytic activities of the four fungal strains after growth under combined conditions (20 d), in medium containing filter paper (FP), sawdust (SW), or sugar cane bagasse (SCB) as the carbon sources. Enzyme activities: CMCase (C), avicelase (A), xylanase (X), and β-glucosidase (G).

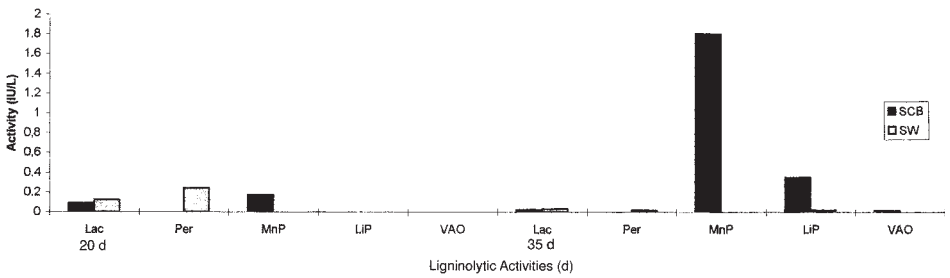

Fig. 6. Ligninolytic activities during growth of *T. canadense* (Q10) under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing sugar cane bagasse (SCB) or sawdust (SW) as the carbon sources. Enzyme activities: laccase (Lac), peroxidase (Per), manganese peroxidase (MnP), lignin peroxidase (LiP), and veratryl alcohol oxidase (VAO).

Low levels of ligninolytic activities were detected in the culture supernatants of strains Q10, H2, LH5, and F20 after their growth in sugar cane bagasse or sawdust under microaerophilic conditions (Figs. 6–9). Regardless of the strain or the carbon source, MnP activity, which was shown by all four strains, was the highest ligninolytic activity produced. Growth under combined conditions (Fig. 10) did not seem to have a positive effect on the production of these enzymes, since, in general, these activities were much lower than the ones detected after their growth at the low oxygen concentration present under the microaerophilic conditions.

Fermentation Products

The production of ethanol and other useful chemical feedstocks from lignocellulosic materials by direct fermentation is more complex than from isolated forms of cellulose or hemicellulose, owing to the presence of lignin, the crystallinity of cellulose, as well as the presence of a complex mixture of sugars. However, the four fungal strains were able to ferment both ligno-

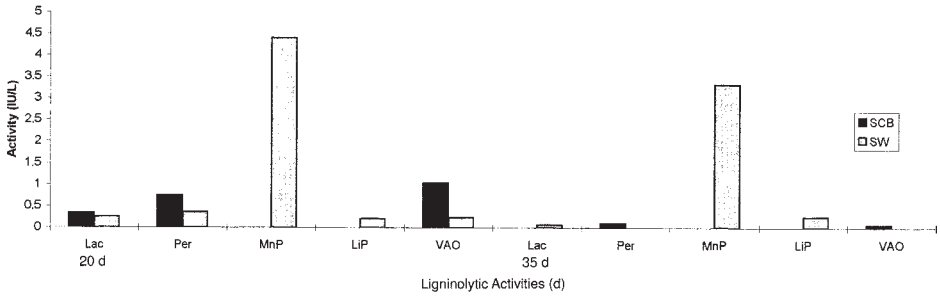

Fig. 7. Ligninolytic activities during growth of strain H2 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing sugar cane bagasse (SCB) or sawdust (SW) as the carbon sources. Enzyme activities: laccase (Lac), peroxidase (Per), manganese peroxidase (MnP), lignin peroxidase (LiP), and veratryl alcohol oxidase (VAO).

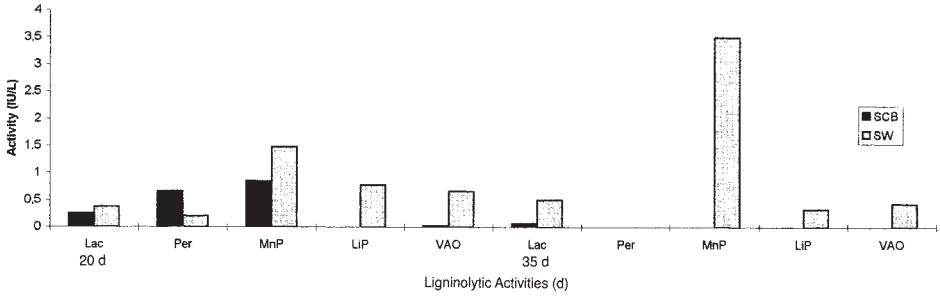

Fig. 8. Ligninolytic activities during growth of strain LH5 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing sugar cane bagasse (SCB) or sawdust (SW) as the carbon sources. Enzyme activities: laccase (Lac), peroxidase (Per), manganese peroxidase (MnP), lignin peroxidase (LiP), and veratryl alcohol oxidase (VAO).

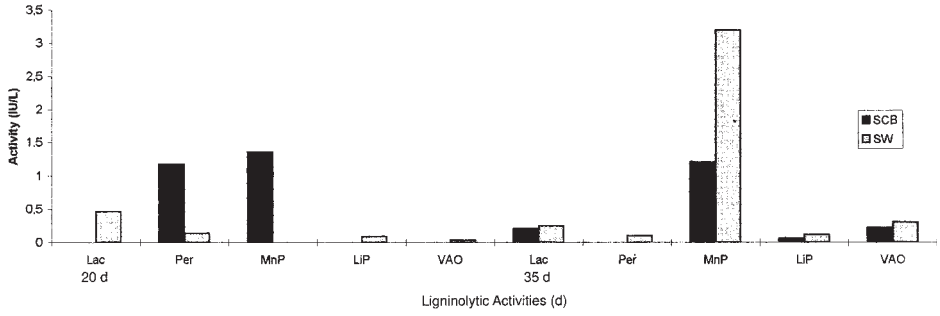

Fig. 9. Ligninolytic activities during growth of strain F20 under microaerophilic conditions for 20 and 35 d at 30°C, in medium containing sugar cane bagasse (SCB) or sawdust (SW) as the carbon sources. Enzyme activities: laccase (Lac), peroxidase (Per), manganese peroxidase (MnP), lignin peroxidase (LiP), and veratryl alcohol oxidase (VAO).

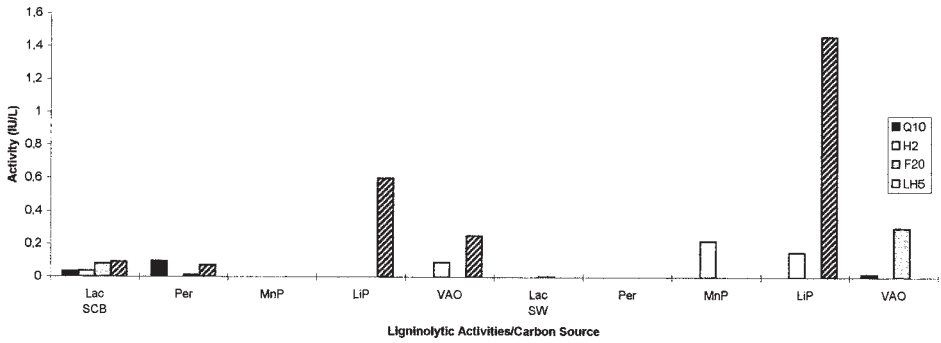

Fig. 10. Ligninolytic activities of the four fungal strains after growth under combined conditions (20 d), in medium containing sugar cane bagasse (SCB) or sawdust (SW) as the carbon sources. Enzyme activities: laccase (Lac), peroxidase (Per), manganese peroxidase (MnP), lignin peroxidase (LiP), and veratryl alcohol oxidase (VAO).

Table 1
Nongaseous Fermentation Products Detected in Culture Supernatants of the Four Fungal Strains After Their Growth Under Microaerophilic Conditions

Carbon source	Microorganism	Fermentation products
Avicel	H2, Q10, and LH5	Ethanol, acetate, and citrate
Xylan	H2, Q10, and LH5	Ethanol, acetate, and malate
Bagasse	H2, Q10, LH5, and F20	Ethanol, acetate, and formate
Sawdust	H2, Q10, LH5, and F20	Ethanol, citrate, formate, and malate
Filter paper	H2, Q10, LH5, and F20	Ethanol, acetate, citrate, and formate

cellulosic substrates used in this study. Table 1 shows that ethanol, formate, acetate, citrate, and malate were the main fermentation products produced by most of the four strains after growth under microaerophilic conditions.

When growing on filter paper medium under stationary conditions, ethanol, acetate, citrate, and malate were detected in the supernatant samples of the four strains. Under shaken conditions, however, citrate and malate were the only products detected in the growth medium of the four fungal strains.

Substrate Utilization

As shown in Table 2, in addition to cellulose, all the isolates used D-glucose, D-fructose, D-mannose, D-galactose, and D-xylose. Strains F20 and H2 presented best growth in most of these sugars.

All the strains tested were able to grow on filter paper, xylan, Avicel, sawdust, and sugar cane bagasse under microaerophilic and combined conditions. Strains H2 and F20 exhibited the best activities of CMCase, avicelase, and xylanase. Strain LH5, on the other hand, showed the best activity of β-glucosidase. The results obtained with *T. canadense* (Q10),

Table 2
Substrate Utilization by the Four Fungal Strains[a]

Strain	Glucose	Fructose	Xylose	Arabinose	Mannose	Galactose
H2	++	+++	+++	++	++	++
Q10	++	++	++	+	++	+
F20	++	+++	+++	+++	+++	++
LH5	+	++	+	+	+	+

[a]Growth was assessed as follows: +++, good; ++, moderate; +, weak.

H2, and LH5 suggest that they prefer a low oxygen concentration for growth and degradation of lignocellulosic materials.

The ligninolytic activities produced under low oxygenation conditions suggest that these strains may be able to attack the lignin component of plant cell walls, not only under aerobic conditions but also under lower levels of oxygenation. Tuor et al. *(24)* have reported that the gaseous regime within the wooden substrate influences enzyme activities of white-rot fungi and the selectivity of lignin biodegradation in vivo. They have also suggested the possibility of in vivo lignin degradation occurring under microaerobic conditions. In this case, low nitrogen concentration, high carbon dioxide, low oxygen partial pressure, and high humidity would be the most important environmental factors for in vivo lignin biodegradation. It is possible that a similar environment has been established in the sealed jars used for microaerobic growth of the fungal strains. Under this condition, strains H2, Q10, LH5, and F20 were able to grow, produce lignocellulases, and also directly ferment some of the monosaccharides produced since, in addition to ethanol, acetate and organic acids were detected in these culture supernatants.

In view of their ability to produce enzymes necessary for the breakdown of lignocellulosic materials, and to utilize most of the degradation products for growth and fermentation, strains H2, Q10, LH5, and F20 have potential for biotechnological application.

Acknowledgments

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References

1. Wyman, C. E. (1994), *Bioresour. Technol.* **50,** 3–16.
2. Wyman, C. E. (1996), in *Handbook on Bioethanol: Production and Optimization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, pp. 1–18.
3. Philippidis, G. P. (1996), in *Handbook on Bioethanol: Production and Optimization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, pp. 253–286.
4. Bath, M. K. and Bath, S. (1997), *Biotechnol. Adv.* **15,** 583–620.

5. Beguin, P. (1990), *Annu. Rev. Microbiol.* **44,** 219–248.
6. Coughlan, M. P. (1992), *Bioresour. Technol.* **39,** 107–115.
7. Beguin, P. and Aubert, J. P. (1994), *FEMS Microbiol. Rev.* **13,** 25–58.
8. Himmel, M. E., Adney, W. S., Baker, J. O., Nieves, R. A., and Thomas, S. R. (1996), in *Handbook on Bioethanol: Production and Optimization*, Wyman, C. E., ed., Taylor & Francis, pp. 143–162.
9. Brigham, J., Adney, W. S., and Himmel, M. E. (1996), in *Handbook on Bioethanol: Production and Optimization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, pp. 119–142.
10. Janshekar, H. and Fiechter, A. (1983), *Adv. Biochem. Eng./Biotechnol.* **27,** 119–178.
11. Kaal, E. J., De Jong, E., and Field, J. A. (1993), *Appl. Environ. Microbiol.* **59,** 4031–4036.
12. Lee, J. (1997), *J. Biotechnol.* **56,** 1–24.
13. Hackett, W. F., Connors, W. J., Kirk, T. K., and Zeikus, J. H. (1977), *Appl. Environ. Microbiol.* **33,** 43–51.
14. Zeikus, J. G. (1990), in *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*, vol. 1, Kirk, T. K., Higuchi, T., and Chang, H.-M., eds., CRC Press, Boca Raton, FL.
15. Blanchette, R. A. (1991), *Annu. Rev. Phytopathol.* **29,** 381–398.
16. Jensen, K. F. (1969), *Enz. Sci.* **15,** 246–251.
17. Schneider, H. (1989), *CRC Crit. Rev. Biotechnol.* **9,** 1–40.
18. Singh, A., Kumar, P. K., and Schuger, K. (1992), *Biotechnol. Appl. Biochem.* **16,** 296–302.
19. Durrant, L. R., Canale-Parola, E., and Leschine, S. B. (1995), in *The Significance and Regulation of Soil Biodiversity*, Collins, H. P., Robertson, G. P., and Klug, M. J., eds., Kluwer Academic, Dordrecht, Netherlands, pp. 161–167.
20. Miller, G. L. R., Blum, R., Glennon, W. E., and Burton, A. L. (1960), *Anal. Biochem.* **2,** 127–132.
21. Tien, M. and Kirk, T. K. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 2280–2284.
22. Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984), *FEBS Lett.* **169,** 247–250.
23. Szklark, G. D., Antibus, R. K., Sinsabaugh, R. L., and Linkins, A. E. (1989), *Mycology* **81,** 234–240.
24. Tuor, U., Winterhalter, K., and Fiechter, A. (1995), *J. Biotechnol.* **41,** 1–17.