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Growth of Lignocellulosic-Fermenting Fungi on Different Substrates Under Low Oxygenation Conditions

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Abstract

Four soil fungi able to grow under low oxygenation conditions were selected and used in studies to determine the production of enzymes that promote the degradation of lignocellulosic materials. The capacity of these fungi to ferment such materials was also investigated. The fungi were grown in sugarcane bagasse and sawdust at final concentrations of 4 and 10%, as the carbon sources. The strains were cultivated under microaerophilic and combined conditions of oxygenation (aerobic followed by microaerophilic conditions). The results obtained with the basidiomycete specie, *Trichocladium canadense*, *Geotrichum* sp., and *Fusarium* sp. suggest that they prefer lower oxygen concentration for growth and enzyme production. Lignocellulolytic activities were detected in all strains but varied with the carbon source used. The highest levels of these activities were produced by the Basidiomycete specie and *Fusarium* sp. 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. 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, these strains have a great potential for biotechnological application.

Index entries: Biodegradation; lignocellulosic fermentation fungi; microaerophilic.

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Introduction

In nature, lignocellulose accounts for the major part of biomass, and, consequently, its degradation is essential for the operation of the global carbon cycle. Lignocellulose, such as wood, is mainly composed of a mixture of cellulose (40%), hemicellulose (20–30%), and lignin (15–25%). 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 source of biomass . For many types of lignocellulosic material, xylan, a polymer of xylose, represents the predominant fraction of the hemicellulose component. The third largest fraction 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)*. Therefore, the degradation of cellulosic biomass represents an important part of the carbon cycle within the biosphere. Apart from its special significance in the biologic carbon cycle, it has enormous potential as an alternative source of fuel, feedstock for the chemical industry, and conversion to edible biomass *(3)*.

Many studies have been conducted to find a more economic application for the native cellulosic wastes, which represent >60% of agricultural wastes *(2)*. Enormous amounts of agricultural, industrial, and municipal lignocellulose wastes have been constantly piled up or used quite inefficiently, because of the very high cost of their utilization processes. This becomes a problem of primary importance for the ecology and for the chemical and biotechnological industries. Therefore, there is a considerable economic interest in the development of processes for effective pre-treatment and utilization of cellulosic wastes as inexpensive carbohydrate sources *(3)*.

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

Advances in industrial biotechnology offer potential opportunities for economic utilization of agroindustrial residues such as sugarcane bagasse, which is a complex material and is the major byproduct of the sugarcane industry. Because of its abundant availability, it can serve as an ideal substrate for the microbial process for the production of value-added products *(5)*.

Cellulose is degraded by an association of enzymes acting in a concerted fashion. This cellulase enzyme system consists of three major components, referred to as endoglucanase (carboxymethylcellulase, [CMCase]), exoglucanase (avicelase), and cellobiase (β-glucosidase) *(6–7)*. 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 the white-rot fungi, which cause the greatest degree of mineralization, best exemplify them. Lignin is degraded during secondary metabolism, which is triggered by a 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 (Lac), and H_2O_2-producing oxidase *(12)*.

Most of the lignocelluloses in nature are 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)*. Oxygen concentrations affect lignin degradation and the greatest ligninolytic activities have been associated with high oxygen concentrations. However, oxygen concentrations within tree trunks are extremely low, and decomposing logs in a rain forest would not be expected to have an environment of high oxygen. Since ligninolytic fungi growing in these logs are involved in lignin degradation, they must pocess a mechanism that is operative under such conditions *(14)*. The microbial production of chemicals by lignocellulose fermentation has generated considerable research interest, and production from biomass in the form of agricultural and forest residues is attractive as an alternative renewable resource *(15)*.

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

Materials and Methods

Microorganism

Four strains of cellulolytic fungi were used. Strains *Trichocladium canadense* (Q10), (ATCC 201360) and the basidiomycete specie (H2) were isolated under anaerobic conditions as described by Durrant et al. *(16)*. Strains *Geotrichum* sp. (LD) and *Fusarium* sp. (FUS) were isolated from soil samples collected near Campinas, Sao Paulo, Brazil.

Media and Culture Conditions

The four fungal strains were cultivated in various carbon sources, such as sawdust and sugarcane 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-cystein hydrochloride, 1.0 mL of resazurin solution (0.1% [w/v]), 0.22 g/L of a 1.0-mL mineral solution containing 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, 10 mg/ of aluminium 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 4.0 and 10.0% (dry w/v). Inoculated erlenmeyer flasks (50 mL of medium/100-mL flask) were incubated under both microaerophilic and combined conditions at 30°C. 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, Brazil). These plates generate a reduced oxygen (5–15% O_2), enriched carbon dioxide (10% CO_2) environment within the incubation jars. Throughout 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. 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 opening of the jars. For the combined conditions inoculated flasks were first incubated under shaking at 150 rpm followed by incubation under microaerophilic conditions: 14 d under shaking followed by 7 d under microaerophilic conditions, 7 d under shaking followed by 14 d under microaerophilic conditions, and 7 d under shaking followed by 21 d under microaerophilic conditions. The supernatant fluids were collected via filtration and used for the determination of enzyme activities and fermentation products.

Enzyme Preparation and Assays

CMCase and avicelase activities were determined by measuring the production of reducing sugars from either carboxymethylcellulose (sodium salt, low viscosity; Sigma, St Louis, MO) or Avicel, by means of the dinitrosalicylic acid method *(17)*. β-glucosidase activity was assayed using ρ-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. All enzyme activities were expressed in units per liter; with one unit equal to 1 μmol of substrate oxidized/min.

LiP, MnP, and Lac activities were assayed spectrophotometrically in the extracellular fluid of the culture supernatants. LiP was assayed by the method of Tien and Kirk *(18)* with veratryl alcohol as a substrate. MnP was assayed by measuring oxidation of phenol red *(19)*. Lac and peroxidases were determined with syringaldazine as the substrate *(20)*. All enzyme activities were expressed in units per liter; with one unit equal to 1 µmol of substrate oxidize/min.

Determination of Soluble Fermentation Products

Following growth, the culture filtrates were assayed by high-performance liquid chromatography (HPLC) to determine the presence of fermentation products, such as acetate, citrate, ethanol, and lactate. An HPLC system (Shimadzu) consisting of an LC-6A pump, a CTO-6AS oven, an SCL-6B controller, an SPD-6A ultraviolet (UV)/visible detector, an RID-10A refractive index detector, and Chromatopac CR-4A software for analytical data storage was used. A Supelcogel C610H ion-exclusion column (300 × 7.8 mm id) was used, and separation was carried out utilizing an isocratic elution with 0.1% (v/v) phosphoric acid in deionized water. The flow rate of the eluent was 0.6 mL/min and the column temperature was 40°C. The uv detector was set at 210 nm for all acids, according to the manufacturer's instructions (Supelco). All the liquid samples were simply filtered through a 0.45-µm syringe-tip filter (Millipore, Bedford, MA) and injected directly onto the column.

Determination of Fungal Biomass

The four fungal strains were cultivated in sawdust and sugarcane bagasse as the carbon sources. After growth, the formation of biomass was estimated by monitoring the increase in mycelial protein by the method of Messner and Kubicek *(21)*.

Results

Determination of Enzyme Activities

As shown in Tables 1–8, the four fungi grew and produced lignocellulolytic enzymes under combined shaking followed by microaerophilic conditions. The best activity for CMCase was produced by strain FUS (~1140 IU/L) in sawdust medium containing sawdust (4%) as the carbon source (14 d under shaking followed by 7 d under microaerophilic conditions). Strains FUS and Q10 (~1100 IU/L) were the best producers of avicelase, mainly in medium containing sawdust (10%), as the carbon source after 20 d under microaerophilic conditions. The best activity of β-glucosidase was produced by strain Q10 (~811 IU/L) in medium containing sawdust (10%) as the carbon source (14 d under shaking followed by 7 d under microaerophilic conditions).

Table 1
Cellulolytic Activities (IU/L) During Growth of Strain H2 Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions [a]

Microaerophilic Conditions	Carbon Source							
	4% Sugarcane bagasse		10% Sugarcane bagasse		4% Sawdust		10% Sawdust	
	20 d	30 d	20 d	30 d	20 d	30 d	20 d	30 d
CMCase	570	200	1000	1000	500	500	500	500
Avicelase	230	250	500	500	500	600	800	700
β-glucosidase	6	6	30	10	300	100	10	20

Combined Conditions	Carbon Source											
	4% Sugarcane bagasse			10% Sugarcane bagasse			4% Sawdust			10% Sawdust		
	A	B	C	A	B	C	A	B	C	A	B	C
CMCase	185	952	589	538	651	672	160	273	160	124	394	160
Avicelase	147	283	152	152	214	152	160	170	160	149	188	121
β-glucosidase	10	10	748	17	29	712	17	10	22	19	10	22

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source.

Table 2
Ligninolytic Activities (IU/L) During Growth of Strain H2 Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source							
	4% Sugarcane bagasse		10% Sugarcane bagasse		4% Sawdust		10% Sawdust	
	20 d	30 d	20 d	30 d	20 d	30 d	20 d	30 d
LiP	4.50	8.50	4.20	0.89	0.02	0.07	0.80	0.44
Lac	0.20	0.15	0.05	0.05	0.05	0.10	0.30	0.80
Peroxidase	0.40	0.28	0.17	0.05	0.05	0.05	0.50	0.60
MnP	1.40	0.70	0.35	ND	2.90	ND	4.30	1.10

Combined Conditions	Carbon Source											
	4% Sugarcane bagasse			10% Sugarcane bagasse			4% Sawdust			10% Sawdust		
	A	B	C	A	B	C	A	B	C	A	B	C
LiP	0.07	ND	ND	0.95	7.50	2.22	0.17	0.38	0.42	ND	0.14	ND
Lac	1.02	0.22	1.04	0.78	0.08	0.71	0.84	0.10	1.12	0.73	0.69	0.51
Peroxidase	1.09	0.61	0.88	0.91	0.07	0.81	0.79	0.29	0.84	0.85	0.29	0.62
MnP	ND	ND	ND	ND	ND	0.05	ND	1.37	ND	ND	0.56	ND

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source. ND, not detected.

Table 3
Cellulolytic Activities (IU/L) During Growth of Strain Q10 Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
CMCase	180	170	170	150	300	640	1100	540
Avicelase	250	170	350	300	400	600	1100	560
β-glucosidase	300	600	400	500	550	600	200	200

Combined Conditions	Carbon Source: 4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
CMCase	147	152	158	150	188	150	147	142	152	150	163	145
Avicelase	152	152	158	168	152	147	140	142	152	147	142	145
β-glucosidase	45	23	15	27	6	13	613	783	772	811	793	793

[a](A)14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C)7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4% or 10% sawdust as the carbon source.

Table 4
Ligninolytic Activities (IU/L) During Growth of Strain Q10 Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
LiP	1.57	1.75	5.80	1.00	1.00	1.40	0.90	0.10
Lac	0.20	0.60	0.15	0.81	0.05	0.10	0.70	0.80
Peroxidase	0.50	0.70	0.20	0.86	0.05	0.05	0.50	0.40
MnP	ND	0.90	ND	2.00	0.80	0.90	0.35	0.70

Combined Conditions	Carbon Source: 4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
LiP	0.02	ND	ND	ND	0.36	ND	ND	ND	0.12	ND	1.27	0.07
Lac	0.24	0.61	0.49	0.51	0.58	0.62	0.39	0.43	0.46	0.40	0.66	0.36
Peroxidase	0.24	0.79	0.48	0.53	0.33	0.67	0.35	0.63	0.45	0.40	0.33	0.34
MnP	ND	ND	ND	ND	0.62	1.96	ND	0.22	ND	ND	0.09	ND

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source. ND, not detected.

Table 5
Cellulolytic Activities (IU/L) During Growth of Strain FUS Under Microaerophilic Condition for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
CMCase	170	500	190	170	220	350	610	160
Avicelase	200	200	370	350	420	320	1100	620
β-glucosidase	500	700	540	440	340	560	640	330

Combined Conditions	4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
CMCase	142	677	142	163	484	163	1140	1138	1130	1110	1114	1114
Avicelase	290	210	250	240	160	230	190	160	310	330	160	300
β-glucosidase	8	6	2	5	190	140	80	550	790	780	790	760

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source.

Table 6
Ligninolytic Activities (IU/L) During Growth of Strain FUS Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
LiP	1.20	1.40	0.35	9.48	1.00	7.00	0.16	0.85
Lac	0.50	0.20	0.10	0.50	0.20	0.05	0.70	1.00
Peroxidase	0.60	0.05	0.70	0.55	0.05	0.05	0.50	0.20
MnP	3.00	2.49	0.10	0.50	3.59	1.15	3.80	0.24

Combined Conditions	4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
LiP	ND	ND	ND	0.53	ND	ND	0.23	0.07	0.06	1.10	7.42	0.40
Lac	0.29	0.53	0.98	0.30	0.52	0.70	0.12	0.62	0.28	0.18	0.63	0.34
Peroxidase	0.77	0.73	1.12	0.48	0.41	0.66	0.51	0.70	0.66	0.29	0.35	0.65
MnP	ND	ND	ND	ND	ND	2.58	ND	0.85	ND	ND	1.51	ND

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source. ND, not detected.

Table 7
Cellulolytic Activities (IU/L) During Growth of Strain LD Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
CMCase	150	150	150	150	140	150	150	160
Avicelase	180	170	320	270	380	330	360	320
β-glucosidase	10	10	10	10	10	10	10	10

Combined Conditions	Carbon Source: 4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
CMCase	320	140	300	270	190	290	630	150	620	160	270	160
Avicelase	369	152	348	327	142	327	214	147	152	163	142	173
β-glucosidase	22	22	22	22	22	22	22	22	22	22	22	22

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source.

Table 8
Ligninolytic Activities (IU/L) During Growth of Strain LD Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
LiP	0.05	0.57	0.05	0.70	0.04	0.10	0.37	0.35
Lac	0.60	0.80	0.20	0.60	0.70	0.80	0.60	1.00
Peroxidase	0.60	0.66	0.70	0.10	0.30	0.50	0.40	0.50
MnP	4.80	ND	0.44	ND	3.56	1.15	3.80	0.26

Combined Conditions	Carbon Source: 4% Sugarcane bagasse A	B	C	10% Sugarcane bagasse A	B	C	4% Sawdust A	B	C	10% Sawdust A	B	C
LiP	8.10	0.08	0.11	16.00	ND	4.35	0.60	0.09	0.39	0.16	0.60	0.77
Lac	0.69	0.90	0.66	0.50	0.99	0.50	0.70	1.00	0.91	1.24	0.81	0.60
Peroxidase	1.56	1.00	1.56	1.13	0.78	1.15	1.00	1.10	0.97	1.68	0.45	0.90
MnP	ND	ND	ND	ND	0.53	1.96	ND	0.05	ND	ND	1.43	ND

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source. ND, not detected.

Low levels of ligninolytic activities were detected in the culture supernatants of strains Q10, LD, H2, and FUS after growth in sugarcane bagasse or sawdust under combined conditions. The best activity for LiP was produced by strain LD (~16 IU/L) in medium containing sugarcane bagasse (10%) as the carbon source (14 d under shaking followed by 7 d under microaerophilic conditions). Strain LD was the best producer of Lac (~1.24 IU/L) and peroxidase (1.68 IU/L) mainly in medium containing sawdust (10%) as the carbon source (14 d under shaking followed by 7 d under microaerophilic conditions). The best activity of MnP was produced by strain LD (~4.80 IU/L) in medium containing sugarcane bagasse (4%) as the carbon source after 20 d under 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 three fungal strains were able to ferment both lignocellulosic substrates used here. Table 9 shows that acetate, citrate, and lactate were the main fermentation products produced by most of the four strains after growth under combined microaerophilic conditions. Ethanol was produced by strain FUS in medium containing 4 and 10% sawdust as the carbon source after growth under microaerophilic and combined conditions of oxygenation.

Mycelial Protein

Figures 1 and 2 show the biomass production (mycelial protein) during growth of the four fungal strains under microaerophilic and combined conditions, respectively, in medium containing sugarcane bagasse (4 and 10%) and sawdust (4 and 10%) as the carbon source. Highest levels of mycelial protein were produced by strains H2, Q10, and FUS in the medium containing 10% sugarcane bagasse as the carbon source after growth under microaerophilic conditions (20 d); and in medium containing 4% sawdust for strain LD.

Discussion

The ligninocellulytic activities produced under the microaerophilic and combined conditions reported here suggest that these fungal strains may be able to attack the components of plant cell walls, not only under aerobic conditions but also under lower levels of oxygenation. In general, the highest levels of enzyme activities, biomass production, and fermentation products were obtained following cultivation of the fungi under microaerophilic conditions and combined conditions B (7 d under shaking followed by 14 days under microaerophilic growth), followed by combined

Table 9
Fermentation Products Detected in Culture Supernatants of Strains H2, Q10, FUS, and LD During Growth Under Microaerophilic Conditions for 20 d and 30 d and Combined Conditions[a]

Microaerophilic Conditions	Carbon Source: 4% Sugarcane bagasse 20 d	4% Sugarcane bagasse 30 d	10% Sugarcane bagasse 20 d	10% Sugarcane bagasse 30 d	4% Sawdust 20 d	4% Sawdust 30 d	10% Sawdust 20 d	10% Sawdust 30 d
Products								
Acetate	H2	H2, Q10			H2, Q10, FUS	H2, Q10, LD	FUS	
Citrate	H2, Q10, FUS, LD	H2, Q10, FUS, LD	Q10, FUS, LD	H2, Q10, FUS, LD	Q10, FUS	H2, Q10, FUS, LD	H2, Q10, FUS	Q10
Ethanol	H2, Q10			FUS, LD	H2, FUS	H2	H2, Q10, FUS	FUS, LD
Lactate				H2, Q10, FUS	H2, Q10	Q10, LD	FUS	H2

Combined Conditions	Carbon Source: 4% Sugarcane bagasse A	4% Sugarcane bagasse B	4% Sugarcane bagasse C	10% Sugarcane bagasse A	10% Sugarcane bagasse B	10% Sugarcane bagasse C	4% Sawdust A	4% Sawdust B	4% Sawdust C	10% Sawdust A	10% Sawdust B	10% Sawdust C
Products												
Acetate	Q10	Q10	H2, FUS				LD	Q10, LD	LD		H2, FUS	
Citrate	H2	H2, FUS	Q10, LD	H2, Q10, FUS, LD	H2, Q10, FUS, LD	H2, Q10, LD,	LD	H2, FUS, LD	Q10, LD	Q10, FUS, LD	LD	H2, Q10, FUS, LD
Ethanol							FUS	FUS	FUS	FUS	FUS	H2, Q10, FUS
Lactate	H2, FUS, LD	Q10		H2, Q10, FUS, LD		Q10, LD	Q10	Q10			Q10, FUS	Q10, FUS

[a](A) 14 d under shaking followed by 7 d under microaerophilic conditions; (B) 7 d under shaking followed by 14 d under microaerophilic conditions; and (C) 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse and 4 or 10% sawdust as the carbon source. ND, not detected.

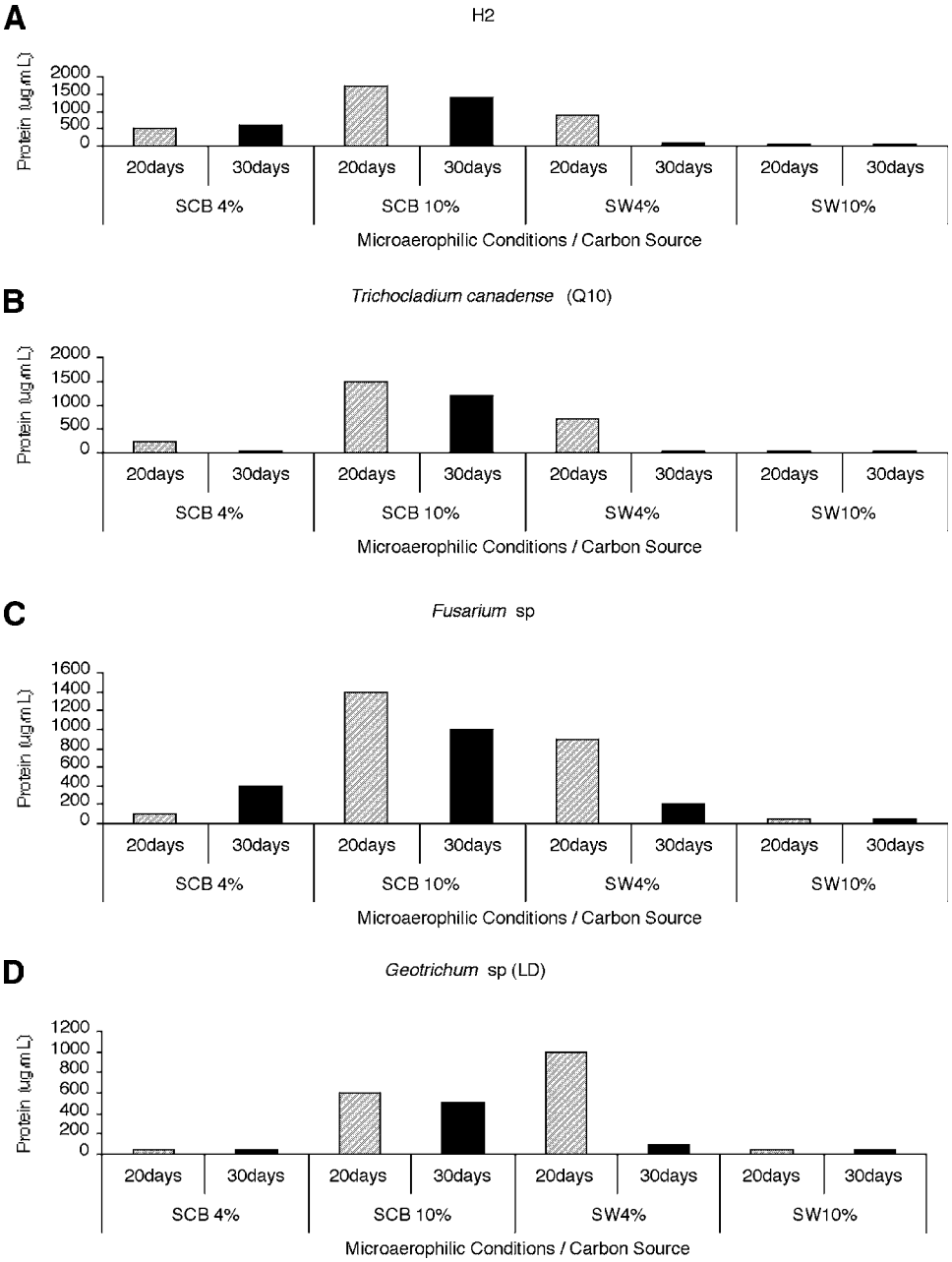

Fig. 1. Mycelial protein (μg/mL) during growth of strains **(A)** H2, **(B)** *T. canadense* (Q10), **(C)** *Fusarium* sp., and **(D)** *Geotrichum* sp. (LD) under microaerophilic conditions for 20 d and 30 d at 30°C, in medium containing 4 or 10% sugarcane bagasse (SCB) and sawdust (SW) 4% or 10% as carbon source.

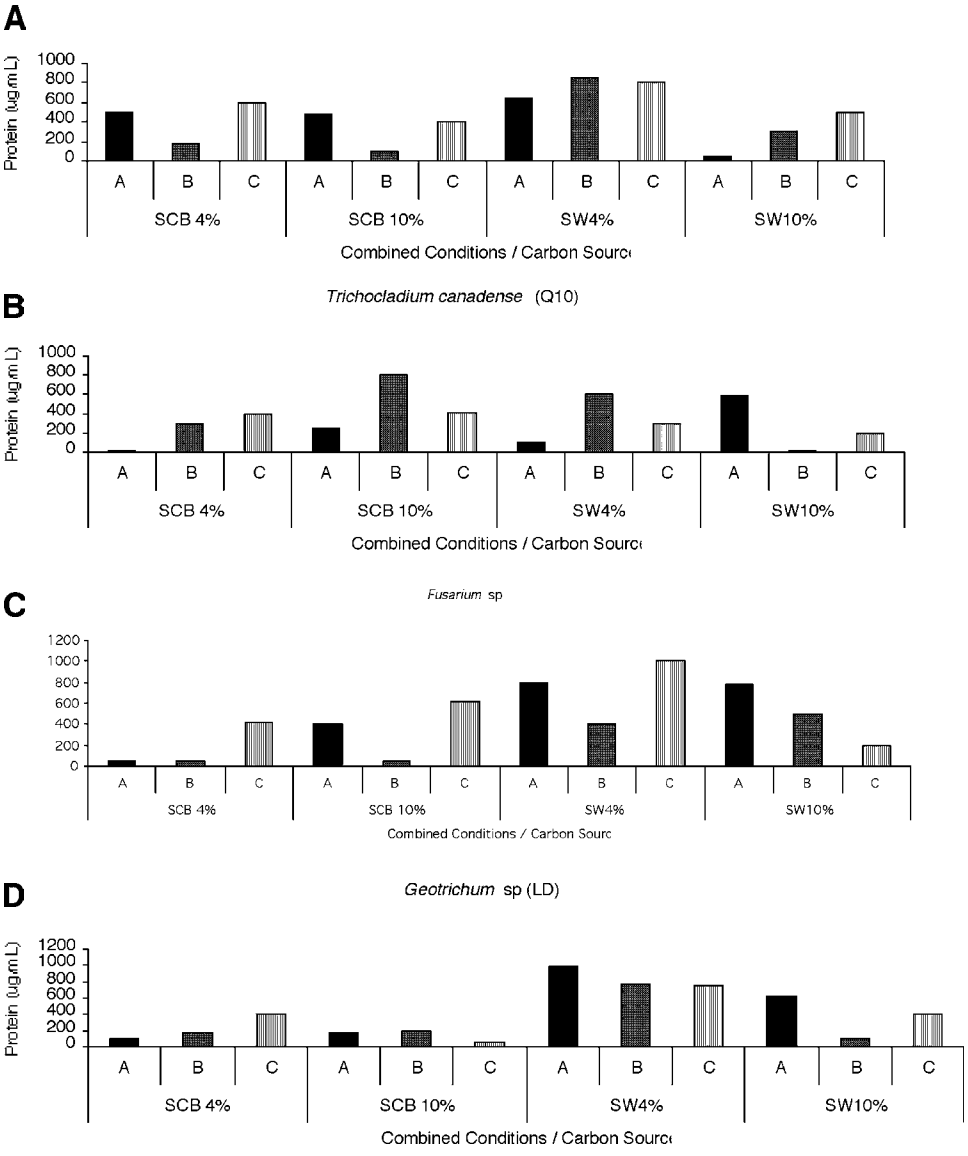

Fig. 2. Mycelial protein (µg/mL) during growth of strains **(A)** H2, **(B)** *T. canadense* (Q10), **(C)** *Fusarium* sp. and **(D)** *Geotrichum* sp. (LD) under combined conditions: A, 14 d under shaking followed by 7 d under microaerophilic conditions; B, 7 d under shaking followed by 14 d under microaerophilic conditions; and C, 7 d under shaking followed by 21 d under microaerophilic conditions, in medium containing 4 or 10% sugarcane bagasse (SCB) and (SW) 4% or 10% sawdust as carbon source.

conditions C (7 days under shaking followed by 21 d under microaerophilic growth), which represent the situations in which the levels of oxygenation during growth were the lowest.

Tuor et al. *(22)* 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 biodegradation of lignin. It is possible that a similar environment has been established in the sealed jars used for microaerobic growth of the fungal strains. Under these conditions, strains Q10, LD, and FUS were able to grow, produce lignocellulytic enzymes, and also directly ferment some of the monosaccharides produced since, in addition to ethanol, acetate and organic acids were detected in these culture supernatants. Durrant et al. *(16)* reported the isolation, using anaerobic culture conditions, of two morphologically and physiologically distinct cellulose-fermenting fungi from soil. Both strains (H2 and Q10) grew and utilized cellulose more rapidly when incubated under microaerophilic conditions, and one strain degraded cellulose most rapidly in well-aerated cultures. Four fungal strains (H2, Q10, LH5, and F20) cultivated under microaerophilic conditions in various carbon sources, such as paper filter, Avicel, xylan, and agroindustrial residues (sugarcane bagasse and sawdust), at a final concentration of 0.5% (dry wt/v) were able to grow, produce cellulolytic and ligninolytic enzymes, and also ferment the substrates *(23)*. The results presented here show that strains Q10, LD and FUS were able to use a much higher concentration of the substrates for growth, which, in turn, had effects on the fermentation pattern and production of the ligninocellulolytic enzymes. While nutrient limitation leads mainly to a decrease in or cessation of metabolic activity, the lack of energy substrates, such as oxygen, forces an organism to switch to a different type of metabolism, or may even cause a shift in the composition of a microbial community *(24)*.

Citrate and lactate followed by acetate were the major fermentations products, following growth of the three strains in sugarcane bagasse and sawdust. Ethanol was only detected in sawdust-grown cultures of strain FUS. Numerous short-chain carboxylic acids are found in liquid media as products of fermentation processes, and chromatographic analyses are the methods of choice to quantify these products. Often a fermentation carried out by one organism (or under one set of growth conditions) will result in a series of organic acid products different from another microbial species (or a different set of growth conditions) *(25)*.

In view of their ability to produce enzymes necessary for the breakdown of lignocellulosic materials, and to utilize most of degradation products for growth and fermentation, the strains H2, Q10, LD, and FUS have potential for the biotechnologic production of biomass, chemicals and fuels.

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