The Production of Hemicellulases by Aerobic Fungi on Medium Containing Residues of Banana Plant as Substrate

R. G. Medeiros,[†] M. L. A. P. Soffner,[‡] J. A. Thomé,[†] A. O. G. Cacais,[†] R. S. Estelles,[†] B. C. Salles,[†] H. M. Ferreira,[†] S. A. Lucena Neto,[†] F. G. Silva Jr.,[‡] and E. X. F. Filho*[,†]

Laboratório de Enzimologia, Departamento de Biologia Celular, Universidade de Brasília, CEP 70910-900, Brasil, and Departamento de Ciências Florestais, ESALQ, Universidade de São Paulo, CEP 13418-220, Brasil

Trichoderma harzianum strains T4 and T6, *Acrophialophora nainiana*, and *Humicola grisea* var. *thermoidea* were screened for their ability to produce carbohydrate-degrading enzyme activities in a medium containing banana plant residue as the carbon source. The best balance of enzyme activities was obtained from cultures of *H. grisea* var. *thermoidea*. Xylanase activity from crude extract of *A. nainiana* had a maximum activity at pH 5.5−7.0 and a temperature range of 50−55 °C. It was stable up to 55 °C at pH 7.0 for at least 2 h. The fungi were also able to produce xylanase and pectinase activities when grown on extractives as substrate.

Introduction

Agricultural and forestry biomasses contain lignocellulosic material available for exploitation as sources of chemical feedstocks, fuels, foods, and feeds (*1*−*3*). In recent years, the use of such materials has also become an alternative approach for the production of cellulose pulp. The banana plant (*Musa cavendishii*) produces a residual component named fruit stalk (*4*). Holocellulose and lignin accounts for as much as 33% and 8.67% of the dry weight of this component, respectively (*4*). Moreover, this residual component produces a high level of extractives containing polyphenols, pectin, and hemicellulose. These extractives are reported to affect the manufacture of pulp and paper, reducing lignin solubility and the yield of kraft pulp and increasing chemical consumption and equipment corrosion (*5*). Therefore, the use of fruit stalk as an alternative source of cellulose for the pulp and paper industry requires the removal of extractives before the pulping process. For bioconversion of these materials it is desirable to have a microorganism capable of producing a variety of enzymes that interact synergistically (*6*). This present work reports the enzymatic hydrolysis of a residual component from the banana plant (fruit stalk) and its extractives by *Trichoderma harzianum* strains T4 and T6, *Acrophialophora nainiana*, and *Humicola grisea* var. *thermoidea*.

Materials and Methods

Enzyme Production. Flasks containing 1.0% (w/v) banana fruit stalk or oat spelt xylan in 100 mL of supplemented medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.06% yeast extract) were inoculated with 1×10^6/mL of spore suspensions from routine subcultures and incubated at 28 °C (*T. harzianum* strains T4 and T6) and 40 °C (*H. grisea* var. *themoidea* and *A. nainiana*) at pH 7.0 for 7 days with shaking at 100 rpm. The content of each flask was filtered through filter paper. The supernatants obtained from the filtration procedure were stored at 4 °C for subsequent use as enzyme assay solutions. The growth on extractive material as substrate was performed as described above in the absence of supplemented medium. Cultures of the above fungi were inoculated with spore suspensions and 50 mL of medium and grown for 8 days, after which samples were taken for determination of mycelial dry weight, reducing sugar, protein content, and xylanase and pectinase activities. The mycelia were washed with distilled water and dried at 95 °C to constant weight. *A. nainiana* was also grown for 15 days in 200 mL of culture medium containing fruit stalk in a concentration range of 0.5−1.5% at the same conditions as described before. Each experiment above was repeated at least three times. The standard deviation was less than ±20% of the mean.

Assays. The hydrolysis of polysaccharides (oat spelts xylan, carboxymethyl cellulose, pectin, and mannan, 1.0%, w/v) *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-α-L-arabinofuranoside, and *p*-nitrophenyl-β-D-mannopyranoside was determined at 50 °C in 50 mM sodium acetate buffer, pH 5.0, as described elsewhere (*7*, *8*). Enzyme activities were expressed as μmol product formed min^{-1} mL^{-1} of enzyme solution, i.e., as IU mL^{-1}. Protein concentration was measured by the method of Bradford (*9*), using bovine serum albumin as the standard. The optimal pH and temperature and stability for xylanase activity from *A. nainiana* crude extract were determined as described before (*8*).

Chemicals. All enzyme assay substrates were purchased from Sigma Chemical Co. (St. Louis, MO). Fruit stalk was from a local source. The extraction of fruit stalk extractives was carried out by refluxing the preparation for 16 h in ethanol, toluene−ethanol (2:1), and distilled water, respectively. The resulting material was evaporated to dryness at 105 ± 2 °C.

* Email: eximenes@unb.br.
[†] Universidade de Brasília.
[‡] Universidade de São Paulo.

10.1021/bp0000398 CCC: $19.00
Published on Web 06/02/2000

Table 1. Some Properties of Crude Extracts from *T. harzianum* Strains T4 and T6, *A. nainiana*, and *H. grisea* var. *thermoidea* When Grown in Different Substrates

fungus	substrate	dry weight (mg/mL)	xylanase activity (IU/mL)	pectinase activity (IU/mL)	reducing sugar (mg/mL)	protein concn (mg/mL)
T. harzianum (T6)	xylan	4.520	6.690	0.520	0.529	0.014
	fruit stalk	nd[a]	5.696	0.363	0.564	0.017
	extractives	0.220	0.133	0.186	0.582	0.016
T. harzianum (T4)	xylan	4.520	3.773	0.603	0.431	0.010
	fruit stalk	nd[a]	2.333	0.625	0.444	0.014
	extractives	0.100	0.343	0.549	0.587	0.015
A. nainiana	xylan	3.978	4.963	0.475	0.271	0.009
	fruit stalk	nd[a]	2.893	0.907	0.332	0.013
	extractives	0.220	0.590	0.505	0.701	0.016
H. grisea	xylan	3.880	5.470	0.588	0.530	0.009
	fruit stalk	nd[a]	6.236	0.700	0.665	0.017
	extractives	0.260	0.480	0.550	0.587	0.016

[a] Not detected.

Table 2. Carbohydrate-Degrading Enzyme Production by Liquid-State Cultures of *H. grisea* var. *thermoidea*, *T. harzianum* Strains, and *A. nainiana* Grown on Fruit-Stalk Substrate

fungus	β-mannanase (IU/mL)	β-xylanase (IU/mL)	β-xylosidase (IU/mL)	α-arabinofuranosidase (IU/mL)	cellulase (IU/mL)
T. harzianum (T4)	0.450	2.97	0.027	0.01	0.145
T. harzianum (T6)	0.464	4.26	0.08	0.01	0.15
A. nainiana	0.512	3.42	0.03	0.035	0.464
H. grisea	1.474	5.18	0.03	0.054	0.978

Results and Discussion

T. harzianum strains T4 and T6, *A. nainiana*, and *H. grisea* var. *thermoidea* were able to grow on medium containing extractives as substrate. The dry weight, reducing sugar, protein content, and xylanase and pectinase activities were compared with those in the medium containing oat spelt xylan or fruit stalk as substrates (Table 1). In all cases, growth of the mycelium was less significant in extractives than in xylan. Unfortunately, it was not possible to determine the dry weight in media containing fruit stalk. Most of the substrate was found to be bound to the mycelium. Extractives did not induce xylanase to an appreciable extent. Low levels of xylanase activity were found in cultures containing pure extractives, A. *nainiana* and *H. grisea* var. *thermoidea* being the best producers. A more pronounced inductive effect of extractives on enzyme activity was obtained for pectinase. Higher amounts of pectinase activity were detected in cultures of *H. grisea* var. *thermoidea*, *A. nainiana*, and *T. harzianum* strain T4. In comparison to the growth on xylan or fruit stalk as substrates, the amount of reducing sugar found with extractives medium was notably higher in *A. nainiana* and *T. harzianum* strain T4. Low amounts of total protein were detected in all cultures.

The above fungi were examined for their ability to produce enzymes capable of breaking down fruit stalk (Table 2). The inductive effect of complex substrates depends on their chemical composition and structure (*10*). Xylan-degrading enzymes (β-xylanase, β-xylosidase, and α-arabinofuranosidase), β-mannanase, and cellulase activities were inducible. The best balance of xylanase activity was found in crude extract samples of *H. grisea* var. *thermoidea* and *T. harzianum* T6. With respect to mannanase, arabinofuranosidase, and cellulase activities, the best yields were obtained with extracts of cultures of *H. grisea* var. *thermoidea* and *A. nainiana*. β-Xylosidase was most active in the extract of a culture of *T. harzianum* T6. Mannosidase activity was not detected in any of the crude extract samples.

The effect of fruit stalk concentration on the production of xylanase activity by *A. nainiana* was examined by adding different concentrations of substrate to the culture media (results not shown). A residual xylanase activity appeared at 24 h. In all cases, a relevant xylanase activity appeared at the 2nd day, suggesting an inductive mechanism. The total protein content was also increased for the same period of incubation. Xylanase activity reached its maximum at the 2nd day and 1.5% fruit stalk and declined to a stable plateau value. The growth at 0.5% and 1.0% fruit stalk behaved differently: xyalanase activity increased throughout the 2nd day of the experiments. The lowest level of enzyme activity was obtained at a fruit stalk concentration of 0.5%. In comparison to the xylanase system from *B. circulans* B_6 (*6*), It seems that the induction of xylanase activity from *A. nainiana* when grown on fruit stalk as substrate is also directly dependent on the substrate concentration present in the medium.

Xylanase activity from a crude extract of *A. nainiana* grown on 1.0% fruit stalk showed a broad pH activity profile and was most active over a pH range of 5.5−7.0. The optimum temperature value was around 50−55 °C at pH 5.0. Xylanase retained 70% of its original activity for at least 2 h at 55 °C and pH 7.0. It dropped to almost 50% and 24% after 3 and 24 h of incubation, respectively.

In conclusion, *T. harzianum* strains T4 and T6, *H. grisea* var. *thermoidea*, and *A. nainiana* produced different levels of carbohydrate-degrading enzyme systems when grown in the presence of fruit stalk and extractives as substrates. These isolates, notably *H. grisea var. thermoidea*, showed a biotechnological potential, especially in the production of cellulose pulp from agricultural residues. Further research on the action mechanisms of these enzyme systems is required to obtain an efficient degradation of banana fruit stalk waste into useful and low-cost products (*11*).

Acknowledgment

This work was supported by a research grant from PADCT III (Brasil). E.X.F.F. and R.G.M. acknowledge receipt of a research fellowship from CNPq (Brasil) and RHAE (Brasil), respectively.

References and Notes

(1) Filho, E. X. F. The xylan-degrading enzyme system. *Brazilian J. Med. Biol. Res.* **1994**, *27*, 1093−1109.
(2) Smith, D. C.; Bhat, K. M.; Wood, T. M. Xylan-hydrolyzing enzymes from thermophilic and mesophilic fungi. *World J. Microbiol. Biotechnol.* **1991**, *7*, 475−484.
(3) Wong, K. K. Y.; Saddler, J. N. Trichoderma xylanases, their properties and applications. In *Hemicellulose and Hemicellulases*; Coughlan, M. P., Hazlewood, G. P., Eds.; Portland Press: London, 1992; pp 171−186.
(4) Rojas, M. L. B. Beneficiamento e polpação da ráquis da bananeira nanicão. Master Thesis, University of São Paulo, Brasil, 1996.
(5) Gardner, J. A. F.; Hills, W. E. The influence of extractives on the pulping of wood. In *Wood Extractives*; Hillis, W. E., Ed.; Academic Press: New York, 1962; pp 367−402.
(6) Kyu, K. L.; Ratanakhanokchai, K.; Uttapap, D.; Tanticharoen, M. Induction of xylanase in *Bacillus circulans* B_6. *Bioresour. Technol.* **1994**, *48*, 163−167.
(7) Silveira, F. Q. P.; Melo, I. S.; Filho, E. X. F. Carbohydrate-hydrolyzing enzyme activity production by solid-state cultures. *Rev. Microbiol.* **1997**, *28*, 152−156.
(8) Ximenes, F. A.; Sousa, M. V.; Puls, J.; Silva, F. G., Jr.; Filho, E. X. F. Purification and characterization of a low molecular weight xylanase produced by *Acrophialophora nainiana* 1999. *Curr. Microbiol.* **1999**, *38*, 18−21.
(9) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(10) Saraswat, V.; Bisaria, V. S. Biosynthesis of xylanolytic and xylan-degrading enzymes in *Melanocarpus albomyces* IIS 68. *J. Ferment. Bioeng.* **1997**, *83*, 352−357.
(11) Krishna, C. Production of bacterial cellulases by solid-state bioprocessing of banana wastes. *Bioresour. Technol.* **1999**, *69* (3), 231−299.

Accepted for publication April 5, 2000.

BP0000398