J. Mycopathol. Res. 44(2) : 289-292 (2006)

Cellulase and ligninase production by white rot fungi associated with bamboo degradation

ARUNA KUNDU AND N. C. CHATTERJEE
Mycology and Plant Pathology Laboratory, Department of Botany, Burdwan University, Burdwan 713104, W. B.

The two wood inhabiting basidiomycetes viz. *Pycnoporus sanguineus* and *Flavodon flavus*, which grow naturally on felled bamboo, were studied for their ability to produce cellulases, ligninases, extracellular phenolases and for their wood degrading ability. Cellulase enzyme activities varied widely in both the species. Cellulolytic enzyme activity remained always higher in bamboo tissue decayed by *F. flavus* (Exoglucanase – 0.1436 U/ml, Endoglucanase – 0.4037 U/ml, and β-glucosidase – 0.3501 U/ml) than the tissues decayed by *P. sanguineus* (Exoglucanase – 0.1287 U/ml, Endoglucanase – 0.3444 U/ml and β-glucosidase – 0.3365 U/ml). Exoglucanase activity was recorded to be significantly less than endoglucanase and β-glucosidase activities. Both the fungi were affirmative of extracellular production of phenol oxidases and were capable of degrading lignin preparation. Through the production of the aforesaid enzymes, both the fungi possess the capacity of degrading bamboo tissues efficiently as visualized by the dry weight loss of the wood tissue *F. flavus* was more efficient degrader of wood tissue than *P. sanguineus* having high cellulase activity.

Key words : *Pycnoporus, sanguineus, Flavodon flavus*, cellulase activity, ligninase activity

INTRODUCTION

Lignocellulosic substances are the most abundant naturally occurring organic polymers in biosphere. The molecular structure of lignocellulose presents a barrier to biodegradation. Efficient and controlled biodegradation of lignocellulosic materials by fungi or bacteria leads to a number of processes of great economic importance. Many microorganism are capable of degrading and utilizing cellulose and hemicellulose as carbon and energy sources, however, a much smaller group of filamentous fungi has evolved with the ability to breakdown lignin, the most recalcitrant component of plant cell walls. Collectively known as white rot fungi, they possess the unique ability to efficiently degrade lignin to CO_2 in order to gain access to the carbohydrate poloymers of plant cell walls for use as carbon and energy sources. These wood decay fungi are common inhabitants of forest litter and fallen trees (Cullen and Kersten, 2004). Potential application of lignin degrading enzymes have received worldwide attention in pretreatment of lignocellulosic substances for the production of liquid and gaseous fuels, biological pulping of paper, fibre bleaching and remidiation of organopollutants (Perumal amd Kalaichelvan, 1996; Cullen, 2002).

Pycnoporus sanguineus and *Flavodon flavus* which are well known white rot basidiomycetes normally grow on dead wooden logs and bamboo, produce wide range of extracellular enzymes to degrade complex lignocellulosic substrate into soluble products. There have been numerous cultural studies on temperate species of wood rotting basidiomycetes (Tresner and Hayes, 1971; Clark *et al*, 1980; Boddy 1986) whereas very few tropical species have been studied in culture. There have also been many studies on decay activities of wood decomposer (Miller,1986; Tanesaka *et al*, 1993) but very little is known about the wood decay abilities of tropical fungi although they are known to cause significant economic damage. In the present study, *in vitro* comparisons have been made of (a) cellulase activity using three different methods (b) phenol oxidase activity (c) ligninase activity and (d) wood decay capacity of *P. sanguineus* and *F. flavus* not previously studied in detail.

MATERIALS AND METHODS

Organisms

Fruit bodies of *Pycnoporus sanguineus* (L. ex.fr.) Murr. (= *Polyporus sanguineus* Fr.) and *Flavodon flavus* (Kl.) Ryv. (= *Irpex flavus*) were collected from felled, decayed bamboo and the pure line cultures of vegetative growth of the fungi were maintained on potato dextrose agar (PDA) slants at 4°C till used.

Measurement of cellulase activity

For cellulase enzyme assay, the cultures (5 mm mycelial agar plug in 100 ml broth) were grown in Basal Salt Vitamin medium. Exoglucanase (FP-ase) activity was determined using Whatman filter paper No 1, following Mandel *et al.*, (1976), Exoglucanase or carboxymethyl cellulase activity was determined following Mandel *et al.*, (1976). β-glucosidase activity was determined using p-nitrophenol β-D- glucopyranoside (*p*NPG). One unit of each enzyme activity was defined as the amount capable of liberating one micromole glucose equivalents released per minute. β-glucosidase activity is defined as the amount of culture filtrate which is capable of liberating one micromole of *p*-nitrophenol per minute from the respective substrates under the condions of assay.

Phenol oxidase tests

The production of extracellular phenol oxidase was tested by dropping an alcoholic solutions of gum guaiacum (0.5 g gum guaiac, Sigma, England, in 30 ml 95% ethyl alcohol)on the mycelial mat of the growing culture. The rapid appearance of blue color indicated the appearance of extracellular oxidase; no change or tardy appearance of pale color indicated a negative result.

Assay for ligninbiodegradation

Alkali lignin and hydrolytic lignin are commercial preparations (Aldrich Chemical Company Inc. Milwakee, USA). The composition of the test agar was as follows - Glucose (5g) NH_4-tartrate (5g), malt extract (1g), $MgSO_4$,$7H_2O$ (0.5g), $CaCl_2$, $2H_2O$ (0.01g), NaCl (0.1g), $FeCl_3$ (0.01g), Vitamin solution (5ml), agar (20g) in 100ml distilled water. A 0.25% (w/v) of the lignin proportion was added to 500 ml of molten hot medium. After thorough mixing the lignin medium was sterilized in the autoclave at 121°C. The pH of the medium was adjusted to 5.5 after which test medium was poured into 9 cm Petri plates. Each isolate was inoculated on a separate test agar plate and incubated at 25°C for 10 days. The points of inoculation were marked on the reverse of the plates. After incubation the growth was scraped off with a razor blade and the plates were flooded with 10 ml. of a reagent consisting of equal parts of 1% (w/v) water solution of $FeCl_3$ and K_3 $[Fe(CN)_8]$ respectively and mixed immediately before use. Strong light was avoided. The test agar is coloured green by this reagent for phenols and the disappearance of lignin is indicated by clear zones around the growth of lignin decomposers.

Assay for biodegradation of wood

The method used to evaluate the wood degrading ability of fungi was based on that of Enoki *et al* (1985). Pieces of bamboo were cut into 1 × 1 × 0.5 cm. One of a pair of blocks cut from adjoining pieces of wood was used in the degradation experiment and the other was used as the control. All wood blocks were extracted with acetone by refluxing for 1 h to remove all soluble wood components. The blocks were then dried in a ventilated oven for 2 h at 70°C. Each wood block was weighed before use. Five days after inoculation of the medium as described above, water soaked bamboo wood blocks were placed on the growing margin of a fungal colony. Wood blocks were removed for analysis after 60 days and 120 days and superficial mycelia were removed with a tooth brush and tap water. Cultural characteristics and wood colour were noted. Each experiment consisted of three replicates per wood treatment and per fungus. The specimens were extracted with distilled water and acetone, dried and weighted.

RESULTS AND DISCUSSION

Cultures were assayed for exoglucanase, endoglucanase and β-glucosidase activities after 10 and 15 days incubation. Cellulase enzyme activity varied widely in both the species and also between

the two sampling times when assays were made (Fig 1). On day 15 endoglucanase and β-glucosidase activity had increased in both the fungi whilst filter paper activity showed the opposite

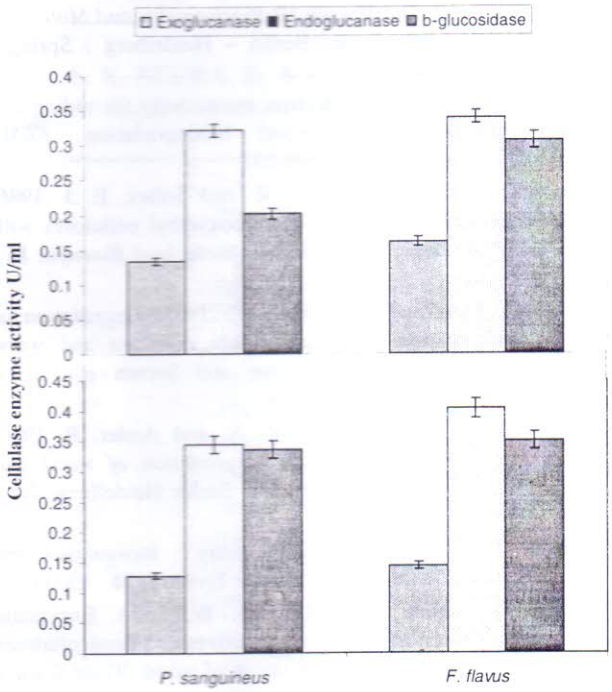

Fig. 1 : Cellulase activity in culture filtrate of *P. sanguineus* and *F. flavus* after 10 days (upper) and 15 days (down) incubation.

trend being reduced in both the fungi on day 15 when compared to day 10. Endoglucanase and β-glucosidase activity were relatively uniform on both sampling days. The fungi were more active against water soluble cellulose derivatives than filter paper cultures. In both the fungi tested filter paper activity was significantly less than endoglucanase and β-glucosidase activities. The highest cellulolytic enzyme activity was expressed by *F. flavus* (exoglucanase – 0.1436 U/ml, endoglucanase – 0.4037 U/ml, and β-glucosidase – 0.3501 U/ml) after 15 days incubation and this activity was also the highest obtained in the whole study. *P. sanguineus* also expressed endoglucanase (0.3444 U/ml), exoglucanase (0.1287 U/ml) and β-glucosidase (0.3365 U/ml) activities on 15 days incubation.

Phenol oxidase and lignin biodegradation assays

Both the fungi gave positive results in the phenol oxidase test and were able to degrade the lignin preparation used. The general rule is that the fungi that produce phenol oxidase cause white rot meaning that they can degrade lignin.

Biodegradation of wood

The 'bamboo' decomposer had widely different wood degrading ability as shown by the weight loss (Fig. 2), *F. flavus* caused 21.50% and 24.38% weight loss of wood whilst *P. sanguineus* cause 23.05% and 30.07% weight loss after 60 days and 120 days incubation respectively.

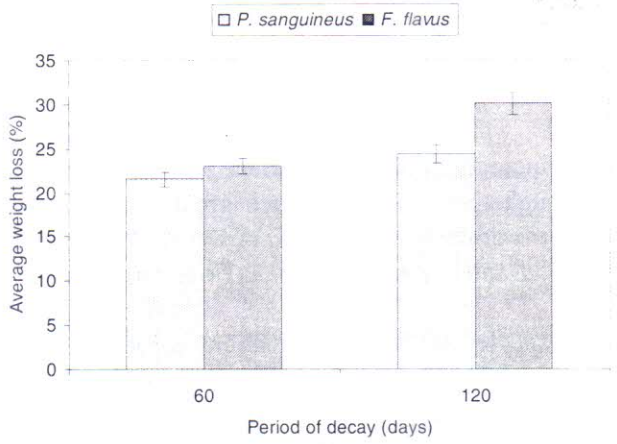

Fig. 2 : Comparison of wood degrading abilities of *P. sanguineus* and *F. flavus* after 60 days and 120 days incubation.

Cellulase enzyme activities varied between the fungi studied. Generally the fungi were more active against water soluble cellulose derivatives than on fitter paper cellulose. This observation could be explained in part by the findings of Dominguez *et al* (1986) showing that carboxymethylation of cellulose significantly enhanced the initial rates of enzymatic hydrolysis of cellulose and increased yields of both reducing sugars and glucose. Purified wood cellulose such as fitter paper retained the rigid three dimensional network characteristics of native cellulose thereby rendering it less susceptible to enzymatic attack. The endogucanase activities recorded in this study were in agreement with Keilich *et al.*, (1970). β-glycosiase activities were generally the same as endoglucanase activities. Study of the whole cellulose complex was estimated using filter paper as a substrate. After 10 days growth both the fungi showed lowering of

exoglucanose (FP-ase) activity. Phenoloxidase reactions were strongly positive in both fungi examined which confirmed that these fungi are white rot.

The versatility of both fungi in their lignolytic abilities is shown by their degradation of the two different lignin preparations used in this study. Lignin is a formidable substrate (Higuchi, 1990, Lewis and Sarkanen, 1998), formed through oxidation and free radical coupling of phenyl alcohol precursors and the insoluble polymer lacks stereoregularity. Extracellular peroxidases and oxidases are thought to play an important role in the initial depolymerization of lignin (Cullen and Kersten, 2004).

Only white rot basidiomycetes have been convincingly shown to efficiently mineralize lignin although species differ in their gross morphological patterns of decay (Erikson *et al.*, 1990; Blanchettee, 1991; Daniel, 1994; Cullen and Kersten, 2004).

The species studied had different wood degrading abilities for degradation of bamboo blocks. *F. flavus* caused higher weight loss than *P. sanguineus*. Through the production of cellulase and ligninase enzymes, both the fungi possess the capacity of degrading bamboo tissue efficiently as visualized by the dry weight loss of the bamboo tissues. *F. flavus* is more efficient degrader of bamboo tissues than *P. sanguineus* having being high cellulase activity.

P. sanguineus and *F. flavus* were isolated predominantly from the bamboo logs and exhibited a particularly strong ability to degrade bamboo tissues. This results corroborate with the findings of Rayner (1977) Shortle and Cowling (1978) and Tanesaka *et al.* (1993).

REFERENCES

Blanchettee, R. 1991, Delignification by wood – decay fungi. *Annu. Rev. Phytopalhol.* **29** : 381-398.

Boddy, L. 1986. Water and decomposition process in water. *Fungi and plants.* (ed P.G. Ayres and L. Boddy) pp 27-48 Cambridge University Press, Cambridge, U.K.

Clark, R. W., Jennings, D. H. and Coggins, C. R. 1980. Growth of *Serpula lachrymans* in relation to water potential substrate. *Tran. Brit. Mycol Soc.* **75** : 271-280.

Cullen, D. 2002. Molecular genetics of lignin degrading fungi and their application in organopollutant degradation. In : Kempken F (ed). *The Mycota* Springer, Berlin Heidelberg. New York. pp. 71-90.

Cullen, D. and Kersten, P. J. 2004. Enzymology and molecular biology of lignin degradation. In : Brambl, R.; Marzluf, G.A., eds. *The Mycota III Biochemistry and Molecular biology*, 2nd edition. Berlin – Heidelberg : Springer Verlag : 249-273.

Daniel, G. 1994. Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiol. Rev.* **13** : 199-233.

Dominguez, G. C., Engler, C. R. and Soltes, E. J. 1986. Enzymatic hydrolysis of carboxyethyl celluloses with varying degrees substitution. *Biotechnol Bioengin* **15** : 99-106.

Enoki, A., Tanaka, H. and Fuse, G. 1985. Degradation of lignin-related compounds, pure cellulose and wood components by white rot and brown rot fungi. *Holzforschung*. **39** : 73-76.

Erikson, K. EL., Blanchettee, R. A. and Ander, P. 1990. *Microbial and enzymatic degradation of wood and wood components*. Springer. Berlin Heidelberg, New York.

Higuchi, T. 1990. Lignin biochemistry : biosynltosis and biodegradation. *Wood Science Technol.* **24** : 23-63

Keilich, G., Bailey, P. and Liese, W. 1970. Enzymatic degradation of cellulose derivatives and hemicelluloses in relation to the fungal decay of wood. *Wood Science and Technol.* **4** : 273-283.

Lewis, N. G., and Sarkanen, S. 1998. *Lignin and lignin biosynthesis. ACS symposium series* 697, ACS, Wasington, DC p.436.

Mandels, M. Andreatti, R. E. and Roche C. 1976. Measurement of saccharifying cellulose. *Biotechnology and Bioengineering Symposium.* **6** : 12-13.

Miller, A. R. 1986. Oxidation of cell wall polysaccharides by hydrogen peroxide : a potential mechanism for cell wall breakdown in plants. *Biochemistry Biophysics Research Communications.* **141** : 238-244.

Perumal, K. and Kalaichelvan 1996. Production of extracellular lignin peroxidase and laccase by *Ganoclerma lucidum* PTK_3 on sugarcane bagasse lignin. *Indian Journal of Experimental Biology*. **34** : 1121-1125.

Rayner, A. D. M. 1977. Fungi colonisation of hardwood stumps from natural sources. *Transaction of the British Mycological Society*. **69** : 303-312.

Shortle, W.C. and Cowling, E. B. 1978. Development discoloration decay and micro organisms following injury of sweet gum and yellow poplar trees. *Phytopathology*. **68** : 609-616.

Tanesaka, E., Masuda, H. and Kinugawa, K. 1993. Wood degrading ability of basidiomycetes that are wood decomposers, litter or mycrohizal symbionts. *Mycologia*. **83** : 347-354.

Tresner, H. D. and Hayes, J. A. 1971. Sodium Chloride tolerance of terristrial fungi. *Appl. Microbiology*. **22** : 210-213.

(Accepted for publication May 30 2006)