J. Mycopathol. Res. **35**(1) :21-28 (1997) Printed in India

Cellulase production by strains *Trichoderma* on water hyacinth biomass

S. MUKHOPADHYAY AND B. NANDI
Department of Botany, Burdwan University, Burdwan - 713104, West Bengal

Cellulase production by six non-mutant strains of *Trichoderma* viz., *T. viride* strains 2109, 3235 and 2185; *T. hamatum, T. reesei and T. lignorum* was studied in liquid still culture containing biomass of water-hyacinth as sole carbon source. Two other substrates, Avicel microcrystalline cellulose and sugarcane bagasse were used for comparison. The culture filtrates were assayed for three cellulase components viz., exoglucanase, endoglucanase and b-glucosidase activity. *Trichoderma viride* 2109 showed highest overall activity followed by *T. hamatum.* All the test fungi produced higher amount of b-glucosidase than exo- and engoglucanase. This feature reflected efficient exoglucanase activity on cellulose. The parallel profile of exo- and endo-glucanase on water hyacinth as well as on Avicel microsrystalline cellulose probably indicated higher degree of crystallinity in water hyacinth imparting resistance to enzyme action as was evident from lower overall enzyme production during earlier days of incubation. Growth pattern of *T. viride* 2109 and *T. hamatum* also proved attractive for their cellulase production.

Key words : Cellulase, *Trichoderma viride, T. hamatum, T, reesei, T. lignosum*

INTRODUCTION

Potentiality of utilizing lignocellulosic wastes for bioconversion to useful products, such as fuel alcohol, organic chemicals, feedstocks by applying cellulolytic enzymes has generated extensive interest during last two decades. In India, increase demand for fuel alcohol arising from alarming population growth and escalating prices for fuel oil has led to renewed interest for exploration of newer lignocellulosic wastes for these purposes.

Water hyacinth, a noxious aquatic weed extensively growing in water bodies of tropic and subtropical regions of the world, poses a manace by clogging lakes, streams, navigable waterways, sewages, lagoons etc. (Zeringue *et al.,* 1979). The vast quantity of this biomass which is available all the year round at no cost in eastern India provides stimulus for the present investigation. During the last decade a few attempts have been made to utilize biomass of water hyacinth as silage for cattle feeding (Biswas and Mandal, 1987; Agarwala, 1987), as feed and manure in carp culture (Mishra *et al.,* 1988; Olha *et al.,* 1990), as substrate for biogas production (Reddy, 1983) or as substrate for mushroom cultivation (Chocooj *et al.,* 1993; Ghosh and Nandi, 1995). However, only a few reports are available regarding its use as carbon source for ethanol production (Kahlon and Kumar, 1987) or for cellulase production (Ali and Akhand, 1992). With this end in view, the present investigation has been undertaken to study suitability of water hyacinth as a substrate for enzymatic hydrolysis or cellulase production.

The fungi of genus *Trichoderma* produce cellulases with all the components required for hydrolysis of crystalline cellulose (Rye and Mandel, 1980). Most of the previous studies on saccharification have utilized cellulases from *T. viride* (Nigam *et al.,* 1987; Patel *el al.,* 1993), *T. reesei,* mainly mutant strains C-30, QM 9414 (Saddler *et al.,* 1985; Lee *et al.,* 1988; Fox *et al.,* 1990; Wayman and Chen, 1992; Gu and Chang, 1992). There are few reports on the use of *T. lignorum* (Patel *et al.,* 1993) and *T. hamatum* (Tan *et al.,* 1986). In present investigation six natural strains of *Trichoderma* have been screened for their cellulase yield, character of individual cellulase component, growth rate and sporulation.

MATERIALS AND METHODS

Microorganisms

Six strains of *Trichoderma*, viz., *T. viride* strains 2109, 3235 and 2185; *T. hamatum* strain 2084; *T. reesei* strain 4026 and *T. lignorum* strain 666 were procured from the type culture collection of Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. Stock cultures of the fungi were maintained on Potato-Dextrose-Agar (PDA) slants and stored at 4°C till used.

Substrates

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms.] belonging to Pontederiaceae, was collected from local ponds after discarding the roots which were reported to contain high quantities of heavy metals (Chocooj *et al.*, 1993). Two other substrates, sugarcane bagasse collected from local market and Avicel microcrystalling cellulose (Sigma) were also used for comparison. Water hyacinth and bagasse were chopped into small pieces, washed thoroughly and oven dried at 60°C for 6 days and then milled to powder (40 mesh) for use as carbon source in flask culture.

Medium and Inoculum

The composition (g/L)of basal medium used in liquid fungal culture was : KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; Urea 0.3; $MgSO_4$. $7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4$. $7H_2O$; 0.005; $MnSO_4$. H_2O, 0.0016 $ZnSO_4$, 0.0014; $CoCl_2$, 0.002; Peptone 1%; Tween-80, 2 ml/L and Yeast extract 0.05 Water hyacinth (3%), sugarcane bagasse (2%) and Avicel cellulose (1%) were added in separate sets to basal media as sole carbon sources to get an equivalent amount of carbon in 1% cellulose in all cases. Initially pH was adjusted to 5 and no further adjustment was done during the incubation period. Erlenmeyer flasks (250 ml) containing 50 ml basal media with substrates were sterilized at 121°C for 20 min., cooled, inoculated with fungal mycelia and incubated at 30 ± 1°C under stationary condition (still culture). Inoculum in each case consisted of five agar discs (7 mm in diameter) which were cut out from the margin of 5 days old growing colony of individual fungus. Cultures were recovered at 4 days intervals and filtered through pre-weighed sintered glass filter (G_2). The filtrates were used for enzyme assays and the biomass were dried at 60°C to constant weights.

Enzyme Asay

The extracellular cellulases in the culture filtrates were assayed for exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and b-glucosidase (EC 3.2.1.21) activities following mainly Mandels *et al.* (1976).

Exoglucanase (FPase) activity was determined by incubating 0.25 ml culture filtrate with 0.75 ml sodium acetate buffer (0.05 M), pH 5, containing 50 mg Whatman No. 1 filter paper discs, at 50°C for 60 mins.

For measurement of endoglucanase (CMCase) activity the reaction mixture contained culture filtrate 0.25 ml, sodium acetate buffer (pH 5) 0.25 ml and carboxymethyl cellulose (1%) in acetate buffer 0.5 ml. It was then incubated at 50°C for 30 mins.

For β-glucosidase activity, 0.25 ml culture filtrate and 0.25 ml acetate buffer were incubated with 0.5 ml of 0.01% cellobiose (Sigma) in sodium acetate buffer at 50°C for 30 mins.

In all cases after specific period of incubations, reactions were terminated by adding 3 ml of dinitrosalicylic acid (DNS) reagent, boiled for 5 mins., cooled to room temperature and the absorbance read at 540 nm. Each enzyme activity was expressed in unit (U) defined as the amount of enzyme required to liberate 1.0 μ mol of reducing sugar (calculated as glucose) per minute under the conditions of assays.

RESULTS

A comparison of the rate of release of reducing sugars was made from filter paper, carboxy methyl cellulose and cellobiose by the culture filtrates of the six test fungi (Figs. 1-3) and they were then screened in terms of yield of those three components of cellulase. *Trichoderma viride* strains 2109, 3235 and *T. hamatum* showed higher cellulase activity on all the substrates than the other strains. However, there were both qualitative and quantitative differences in the enzyme systems of the six strains when grown on different substrates. All the fungi showed much higher (more than double) β-glucosidase activities than those of exo- and endo-glucanases within 4 days of inoculation on water hyacinth. Maximum exoglucanase (0.04 U/ml) and endoglucanase (0.04 U/ml) were produced by both *T. hamatum* and *T. viride* 2109 on bagasse while the minimum activity was by *T. lignorum* on all the substrates. On the basis of summed activities of exoglucanase, endoglucanase and β-glucosidase, on all the three substrates by each individual fungus, they can be placed in a discending order of activities as follows :

T. viride 2109 → *T. hamatum* 2084 → *T. Viride* 3235 →
→ *T. reesei* 4026 → *T. viride* 2185 → *T. lignorum* 666.

No exact correlation could be drawn between biomass and enzyme production though all the carbon sources supported fairly satisfactory mycelial growth.

DISCUSSION

Strains of *Trichoderma,* particularly mutant strains *(e.g. T. reesei* C-30, QM 9414 etc.) are most suitable for practical saccharification because they are known to produce a complete cellulolytic enzyme complex that is capable of total hydrolysis of insoluble cellulose to soluble sugars (Dekker and Wallis, 1983). The six strains used in the present investigation were all naturally occuring and not mutant strains. They were screened to select two best strains for further work on enzymatic hydrolysis of water hyacinth. Pure crystalline Avicel cellulose was incorporated in the study to compare the degree of resistance of water hyacinth towards enzyme action due to presence of crystalline cellulose. Lignocellulosic substrate bagasse which was used by earlier workers (Nigam *et al.*, 1987; River and Emert, 1988; Mitra and Nandi, 1992) was also screened to compare the efficiency of the enzyme towards native lignocellulose.

Earlier evidence suggests that the nature of carbon source plays a strong role in cellulase production (Wood, 1982; Rye and Mandels, 1980). Solubilization of amorphous cellulose can be accomplished by either exoglucanase or endoglucanase independently while hydrolysis of crystalline cellulose results from synergistic interactions of both exo- and endoglucanase (Wood, 1982). Thus, a close relationship exists between substrate and the efficiency of enzyme system to degrade crystalline part of cellulose. The synergistic action of exoglucanase and endoglucanase and more or less parallel profile of their activities on Avicel microcrystalline cellulose (most obvious in *T. viride* 2109) as well as on water hyacinth biomass reflected probably higher amount of crystalline cellulose in the latter. On the other hand, in bagasse each enzyme component reached their maximum level after different periods (within 12-20 days) and then showed wide variations. This differential behavior of enzyme components and earlier production of higher amount of enzyme in bagasse than other substrates might be an indication of the presence of higher amorphous cellulose in bagasse (cf. Mandels *et al.*,1976).

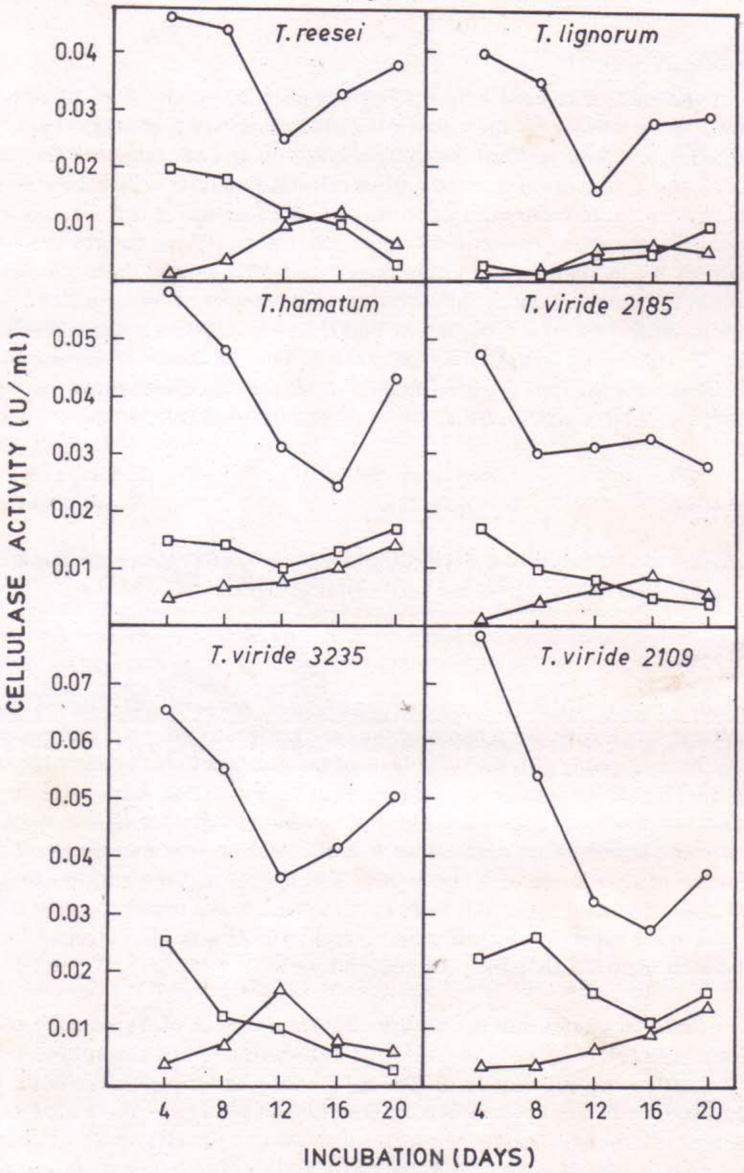

Fig. 1. Cellulase production on the basis of exoglucanase (△—△), endoglucanase (□—□), and β-glucosidase (0—0), activities by six strains of *Trichoderma* on water hyacinth biomass.

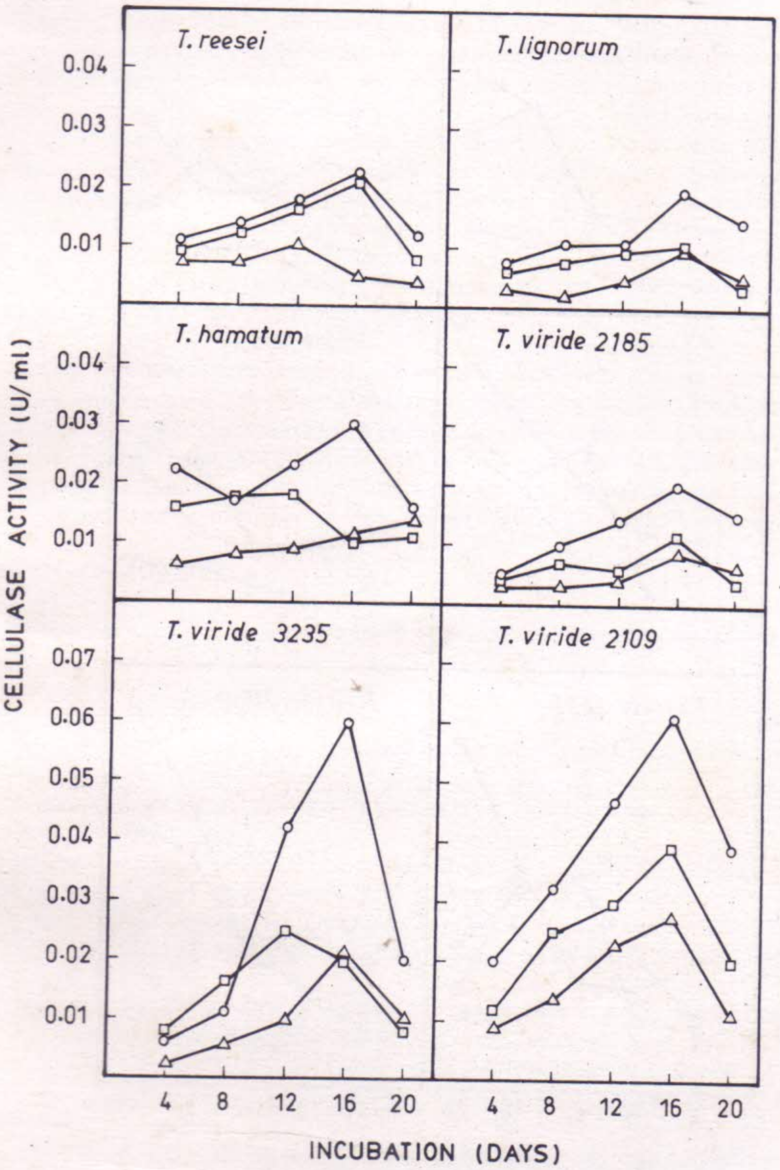

Fig.2. Cellulase production on the basis of exoglucanase (△—△), endoglucanase (□—□), and β-glucosidase (0—0), activities by six strains of *Trichoderma* on Avicel microcrystalline cellulose.

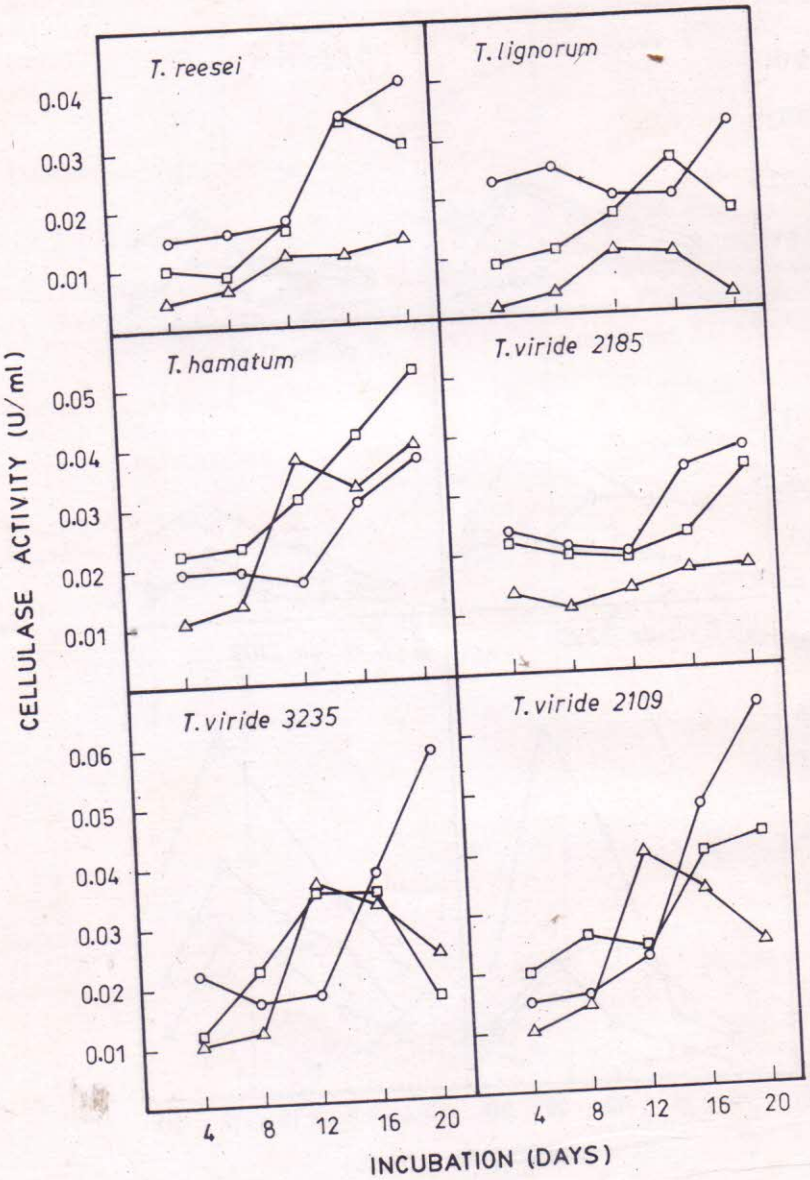

Fig.3. Cellulase production on the basis of exoglucanase (△—△), endoglucanase (□—□), and β-glucosidase (0—0), activities by six strains of *Trichoderma* on sugarcane bagasse.

The most prominent feature in the results of present investigation is the higher β-glucosidase activity of all test species specially on water hyacinth. Although β-glucosidase was reported to have no direct action on cellulose (Sternberg *et al.*, 1977), its high level in an enzymatic mixture prevents accumulation of cellobiose to inhibitory level for exoglucanase activity. Thus, it is considered as an attractive feature of a good cellulase system. Previous works on most efficient mutant strains of *T. reesei* reported problems arising from low β-glucosidase level in an otherwise good cellulase system (Rye and Mandels, 1980; Dekker and Wallis, 1983). To circumvent the defficiency, culture filtrate was often supplemented with separate β-glucosidase source (Sternberg *et al.*, 1977) or whole cultures were used as enzyme source (Gracheck *et al.*, 1980). High β-glucosidase level in culture filtrates from *T. viride* 2109, 3235 and *T.hamatum* was thus considered encouraging. Moreover, substantial amount of endoglucanase (compared to exoglucanase) in those culture filtrates fulfilled the requirement of balance cellulase system. Comparatively lower enzyme activity of the test fungi than other strains of *Trichoderma*, reported earlier, may be attributed to change in pH from 5 to 6.5 during incubation (Gomes *et al.* 1989) and fungal growth in still culture. Further improvement of enzyme production are expected to be achieved through use of agitated culture keeping the pH unaltered during agitation. The poor enzyme activity of *T. lignorum* was evidently resulted from less mycelial growth.

Among the tested organisms *T. viride* strain 2109 and *T.hamatum* showed most rapid, uniform, extensive mycelial growth and sporulation. As lack of homogeneity in mycelial distribution was reported to result in low enzymatic activity, the uniform growth pattern of these two encouraged their selection for further work on water hyacinth. From the present study, the enzymology of cellulolysis of native, unaltered water hyacinth by the non-mutant strains of *Trichoderma* gave useful hints for its effective utilization as a carbon source in fermentation.

ACKNOWLEDGEMENT

The first author thanks University Grants Commission for financial assistance.

REFERENCES

Agarwala, O.N. (1987). Water hyacinth (*Eichhornia crassipes*) silage as cattle feed. *Biol. Wastes*, **25**, 71-73

Ali, M.S. and Akhand, A. A. (1992). Cellulase from *Trichoderma isolate*. *J. basic Microbiol.*, *32(4)*, 259-368.

Biswas, M. and Mandal, L. (1987). Use of wilted water hyacinth *(Eichhornia crassipes)* in ration of growing calves. *Ind. J. Anim. Sci.*, **57((5)**, 482-485.

Chocooj, R. L., Soto-Velnzco, C., Guzman-Davalos, L. and Guzmnn, G. (1993). Cultivation of *Pleurotus* on water hyacinth and determination of the heavy metals in Mexico, *Mushroom Res.*, **2**, 37-40.

Dekker, R.F.H. and Wallis, A.F.A. (1983). Enzymatic saccharification of sugarcane begasse pretreated by autohydrolysis - steam explosion, *Biotech. Bioengg.*, **25**, 3027-3048.

Fox, D. J., Morjanoff, P.J. Gray, P.P., Dunn, N.W, and Marsden. W. L. (1990). Process evaluation for bioconvrsion of sugarcane bagase to component sugars, *Aust. J. Biotechnol.*, **4(1)**, 22-25.

Ghosh, M. and Nandi, B. (1995). Compositional changes of two lignocellulosic materials during degradation by *Pleurotus* spp. in solid state fermentation, *J. Mycopathol. Res.*, **33(1)**, 59-65.

Gommes, J., Gomes, I., Esterbauer, H., Keiner, W. and Steiner, W. (1989). Production of cellulases by a wild strain of *Gliocladium virens*: Optimisation of the fermentation medium and partial characterization of enzymes, *Appl. Microbiol. Biotechnol.*, **31**, 601-608.

Gracheck, S.J., Giddings, K.E., Woodford, L.C. and Emert, G. H. (1980). Continuous enzyme production used in conversion of lignocellulosics to ethanol, In : Agricultural Energy :

biomass energy crop production. Vol. 2. American Society of Agricultural Engineers National Energy Symposium, pp. 305-310.

Gu, Z.Y. and Chang, F.H. (1992). Bioconversion of peat to reducing sugars by multienzyme system of *T. harzianum* and *T. reesei* QM 9414, Abstr, Gen. Meet. Am. Soc. Microbiol. 92 meet, pp. 318.

Kahlon, S. S. and Kumar, P. (1987). Simulation of fermentation conditions for ethanol production from water hyacinth, *Indian J. Ecol.*, **14(2),** 213-217.

Lee, N.E., Lima, M. and Woodward J. (1988). Hydrolysis of cellulose by a mixture of *trichoderma reesei* cellobiohydrolase and *Aspergillus niger* endoglucanase, *Biochemica et Biophysica Acta.*, **967,** 437-440.

Mandels, M., Andreotti, R. and Roche, C. (1976). Measurement of saccharifying cellulases, *Biotech. bioengg. Symp.,* **6,** 21-23.

Mishra, B.K., Sahu, A. K. and Rani, K.C. (1988). Recycling of the aquatic weed, water hyacinth and wastes in raring of Indian Major carps, *Aquaculture,* **68(1),** 59-69.

Mitra, S. and Nandi, B. (1992). Biodegradation of agroindustrial wastes by microorganisms *J. Mycopathol. Res.* **30(1),** 71-79.

Nigam, P., Pandey, A. and Prabhu, K.A. (1987). Mixed cultures fermentation for bioconversion of whole bagasse into microbial protein, *J. Basic microbiol.,* **27(1),** 323-327.

Olah, J., Ayyappan, S. and Purushotanaan, C.S. (1990). Processing and utilization of fermented water hyacinth, *Eichhornia crassipes* (Mart.) Solms. in carp culture, *Aquacult. Hung. no.* **6,** 219-234.

Patel, B., Patel, H. and Kothari, I.L. (1993). Ligninase of *Trichoderma, Int. Congr. Plant pathol.,* **6,** 139.

Reddy, K.R. (1983). Water hyacinth biomass production in Florida, *Sum. South. Biomass Energy Res. Conf.,* 94-95.

Rivers, D.B. and Emert, G.H. (1988). Factors affecting the enzymatic hydrolysis of bagasse and rice straw, *Biol. Wastes,* **26,** 85-95.

Rye, D.D.Y. and mandels, M. (1980). Cellulases : biosynthesis and application, *Enz. microb. Technol.,* **2,** 91-101.

Saddler, J.N., Hogan, C.M. and Louis-Zeize, G. (1985). A comparison between the cellulase systems of *Trichoderma harzianum* E 58 and *T. reesei* C 30, *Appl. microbiol. Biotechnol.,* **22,** 139-145.

Sternberg, D., Vijayakumar, P. and Reese, E.T. (1977). β-glucosidase : Microbial production and effect on enzymatic hydrolysis of cellulose, *Can. J. Microbiol.,* **23,** 139-147.

Tan, T.K., Yeoh, H.H.,and Paul, K. (1986). Cellulolytic activities of *Trichoderma hamatum* grown on different carbon substrates, *Mircen J. Appl. Microbiol. Biotechnol.,* **2(4),** 467-472.

Wayman, M. and Chen, S. (1992). Cellulase production by *Trichoderma reesei* using whole wheat flour, *Enz. Microb. Technol.,* **14,** 825-831.

Wood, T.M. (1982). Enzymatic and other factors affecting the microbial generation of soluble sugars from cellulosic materials, Proceedings of 1982 TAPPI, R & D Conference, 327-334.

Zeringue, S.P., Rusoff, L.L. and Wolverton, B.C. (1979). Water hyacinth - A source of roughage for lactating cows, *J. Dairy Sci.,* **62,** 200 (Proc.).

(Accepted for publication 30 December 1996)