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Cellulolytic activity of some promising fungi in selected biodegradation studies

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Cellulose is the most abundant biological polymer on earth, which is usually cycled through plants and herbivorous animals. Cellulolytic enzymes in the form of biocatalysts secreted by some microbes, especially fungi, play a dynamic role in the conversion of cellulosic biomass into appropriate end product through the process of saccharification and biodegradation. Most of the organic wastes constituting of 40-60% of cellulose are facing a serious disposal problem and these wastes can be profitably utilized by the cellulolytic microbes. Hence our primary aim is to screen some promising fungi, releasing cellulase, a multi-enzyme complex, playing an essential role in saccharification and biodegradation. Lignocellulosic crop residues can be profitably utilized and exploited for the conversion of the biomass to fermentable sugars, which ultimately produce alcohol, fuel, organic acids and antibiotics through fermentation biotechnology. Preliminary screening of the cellulolytic fungi was done by dilution plate and enrichment techniques using 1% carboxy methyl cellulose or filter paper as the sole source of carbon. Fungal colonies obtained in the media are repeatedly streaked on CMC media and finally subcultured on potato dextrose agar slants for microscopic morphological study and proper identification according to standard manual. For the complete saccharification of cellulose to sugar by cellulolytic fungi, participation of all the three components of the enzyme complex, namely endo-β-glucanase (CMCase), exo-β-glucanase (FPase), β-glucosidase, is required. A detailed study on the biosynthesis of the enzymes by these fungi was investigated in standard media. Production of these enzymes by fungi and biodegradation studies with two lignocellulosic substrates under sterilized condition by these organisms were conducted to show the efficiency of cellulolytic fungi in saccharification and biodegradation. The promising fungi screened so far are *Trichoderma viride, T. lignorum Aspergillus japonicus, A. terreus, Penicillum citrinum, P.funiculosum, Chaetomium globosum, Alternaria alternata* etc.

Key words : Cellulolytic mycoflora, lignocellulosic agricultural residue, *Aspergillus japonicus, Penicillium frequentans*

INTRODUCTION

Cellulose is one of the most abundant organic polymers on earth. It contributes to the global carbon cycle and is usually cycled through plants and herbivorous animals. A large number of specialized microorganisms secrete cellulolytic enzymes which act as biocatalyst in conversion of cellulosic biomass into appropriate end-product through the process of saccharification and biodegradation. Most of the organic wastes usually containing of 40%-60% of cellulose are posing a serious disposal problem. During 1985, the production of crop residues viz. wheat straw, rice straw, sugarcane bagasse, leftover from pulses etc. was estimated to be of worth 321 million crores (FAO, 1985). This estimate is only a part of huge quantities of lignocellulosic plant wastes generated every year in India. These wastes can be recycled and profitably utilized by way of conversion of the lignocellulosic crop residues to fermentable sugars and later on to alcohol, fuel, organic acids or antibiotics with the help of modern biotechnological tools. Conversion into sugars and destruction of cellulose-containing substrates has been considered as the key feature of the cellulase. Since enzyme cost is the major impedi-

ment to commercialization of enzymatic cellulose hydrolysis, the major impediment to commercialization of enzymatic cellulose hydrolysis, the major challenge is to significantly increase the activity of cellulases (Walker and Wilson, 1991). Cellulase is a multi-enzyme complex with the participation of all the three components, viz. endo-1,4-β-D glucanase, exo-1,4-β-D glucanase, and endo-1,4-β-D glucanase. Soils being the reservoir of microbial flora, efforts were made to screen different groups of fungi from soil and their utilization in saccharification and biodegradation of various pretreated lignocellulosic crop residues, in our present investigaion. The understanding of fungal cellulases had advanced considerably over the last decade through a combination of biochemical and molecular biological approaches (Goyal *et al.,* 1991).

Modification of lignocellulosic substrates by a variety of physical and chemical manipulations brings about changes in the cellulose lignin complex and also the crystallinity of cellulose ultra structure (Toyama and Ogawa, 1977 ;)

MATERIALS AND METHODS

Collection of sample

Soil

In order to screen cellulolytic fungi as well as to study the biosynthesis of cellulolytic enzyme in standard media and their cellulolytic activities on lignocellulosic agricultural residues for saccharification and biodegradation, soil samples were collected from the rhizospheric regions (15-20 cm) of two crop fields, namely rice and wheat, of I.S.I. Agricultural Research Station, Giridih, Jharkhand.

Lignocellulosic agricultural residues

Two lignocellulosic agricultural residues, namely rice and wheat straw, were also collected from the same field.

Analytical studies

Soil

After collection the soil samples were air dried, sieved through a 3 mm mesh sieve and stored. Physico-chemical analysis of the samples were carried out critically by standard methods for moisture content, pH, organic carbon by Titrimetric method using $K_2Cr_2O_7$, following Walkley Black's method (Jackson, 1973) nitrogen by Macrokjeldahl method (Jackson, 1973), phosphorus using spectrophotometric method with ammonium molybdate and stannous chloride (Black, 1965), sodium using flamephotometeric method with 1(N) ammonium acetate solution (Jackson, 1972), and potassium flame photometeric method using 1(N) ammonium acetate solution (Jackson, 1972).

Lignocellulosic agricultural residues

The lignocellulosic agricultural residues collected were cut into small pieces, air dried, and powdered at the particle size between 20-35 mesh for chemical analysis. Pretreatment of crop wastes (25 g) with 2% NaOH (450 ml) and 121°C in an autoclave (moist heat) was done for an hour. Delignification of lignocellulosic substrates with alkali was suggested by Prof. T. K. Ghosh (personal communication). These samples were thoroughly washed in water and later used as substrate for biodegradation. Total nitrogen, organic carbon, phosphorus and ash analysis were done following standard methods. Other major cell wall constituents like lignin, holocellulose, α-cellulose etc. of the samples were determined following the standard methods (T.A.P.P.I., 1971) with defatted sample.

Microbiological profile study

Screening of cellulolytic fungi

Preliminary screening of the microbes was done using dilution plate and enrichment techniques. Secondary screening was done later using specialized media with 1% carboxy methyl cellulose or filter paper as the sole source of carbon. For fungi, asparigine medium (ammonium sulphate 0.5 g/1, L-asparigine 0.5 g/1, potassium phosphate dibasic 1.0 g/1., magnesium sulphate 0.2 g/1., calcium chloride 0.1 g/1., yeast extract 0.5 g/1., CMC (cellulose 10 g/1., having pH 6.2 was utilized. In addition 0.5% Na propionate was also added. To arrest bacterial growth in fungal medium, Ambistryn-5 (streptomycin sulphate 150 µg/ml.) was incorporated in molten media as a precautionary measure. Total fungal

population (in Czapex-Dox agar medium and PDA) and that of cellulolytic fungi (in asparagine medium) were expressed in terms of colony forming unit (CFU/g.) of dried sample. Frequeney and relative abundance were also calculated according to Clark and Christensen (1981).

2 g of each sample was taken in 500 ml Erlenmeyer flask containing 200 ml CMC (asparagine) medium for enrichment. Triplicate flasks of each isolate after inoculation were incubated in a B.O.D. incubator at 30 ± 2 °C. Every fourth day the contents of the flasks were mixed manually. The cellulolytic fungi were isolated from CMC medium after every 15 days of incubation, followed by dilution plate technique, plated and screened upon CMC-asparagine agar plates.

Identification of cellulolytic fungi

Pure culture of these cellulolytic isolates was finally obtained by repeated streaking on CMC-asparagine medium. Isolates were subcultured on potato-dextrose agar slants for micro morphological studies and proper identification according to Gilman (1957).

Fermentation studies

Fermentation study was done to assess extracellular biosynthesis of celluloytic enzyme using 500 ml Erlenmeyer flask with 100 ml Czapex-Dox agar medium, replacing sucrose with CMC, (CMC, 30 g/1, $NaNo_3$, 2 g/1, K_2HPO_4, 1 g/1, $MgSO_4.\ 7H_2O$, 0.5 g/1, KCl 0.5 g/1, $FeSO_4.\ 7H_2O$, 0.01 g/1) having pH 6.0, and incubated for 9-12 days at 28 ± 1°C. Aliquot was taken and assayed for CMCase, FPase (using 0.5 (M) citrate buffer and DNS) and β-glucosidase (using 0.5 (M) phosphate-citrate buffer and PNPG) activity individually in 540 nm, 540 nm, 420 nm wavelength respectively against reagent blank.

Saccharification studies

Enzyme source

Crude enzyme was obtained for 1000 ml basal medium, inoculum 5% v/v, incubation 8-12 days at rotary shaker. The content of the flask was filtered through glass wool and centrifuged at 5000 rpm. The crude enzyme was precipitated with saturated ammonium sulphate solution (60%) followed by dialysis.

Saccharification

Saccharification was carried out using culture filtrate of *Aspergillus japonicus* and *Penicillium frequentans.* Semipurified enzyme was dissolved in citrate buffer (20 IU/ml FPA). 70 ml enzyme filtrate was added for each flask containing 25 ml of 0.1 M citrate buffer (pH 5.0) and 5 g of NaOH treated waste. The flasks were incubated at 50°C for 72 h in a temperature-regulated waterbath. After incubation the saccharification material was centrifuged at 5000 rpm of 20 minutes and amount of reducing sugar in the syrup was estimated.

$$\% \text{ of saccharification} = \frac{\text{Reducing sugar formed}}{\text{NaOH treated substrate}} \times 0.9 \times 100$$

Biodegradation studies

For biodegradation studies, 3 g of powdered and sterilized lignocellulosic waste was inoculated with 5 ml spore suspension of two cellulolytic fungi, viz. *Aspergillus japonicus* and *Penicillium frequentans* and incubated at 30 ± 2°C in a BOD incubator for 5 days. The infestation of fungal colonies over lignocellulosic residues indicated production of cellulase from 2-5 days. After 2 weeks, cellulose and lignin content were estimated to note the extent of biodegradation.

RESULTS AND DISCUSSION

For the microbiologists, the soil environment is unique in several ways. It contains a vast population of microorganisms and it is the most dynamic site of microbial interactions in nature. Soil microflora has been known to be greatly influenced by soil type and physico-chemical properties of soil (Walksman, 1961).

In our present investigation, the soil samples were collected from rhizospheric regions in search of metabolically active microbes as this area serves as nutrient source for indigenous microflora (Curl and Truelove, 1986). Summarizing the results, the data

in Table 1 show the physico-chemical properties of the soil collected from cultivated fields of Giridih, Jharkhand. Carbon content of rice field soil was comparatively lower than that of the wheat field, nitrogen content being more or less equal. Hence C/N ratio was much higher in case of wheat field. Phosphorus content was slightly higher for wheat field. Again mineral content of both the fields was more or less equal except Na content, which was high in wheat field.

Table 1 : Physiological characteristics of samples collected from cultivated fields of rice and wheat

Source	Location	pH	Moisture	Carbon (C)	Nitrogen (N)	C/N Ratio	P (mg/g)	Na (mg/g)	K (mg/g)
Rice Field	Giridhi	5.8	10.0	0.23	0.16	1.46	0.28	0.03	10.35
Wheat Field	Giridhi	5.6	8.0	2.00	0.17	11.76	0.31	1.66	10.21

Among the entire microflora, fungi have been well accepted as the best cellulose degrader. Hence we have undertaken our present investigation with fungi. Table 2 denotes the population dynamics (CFU/g. dry soil or substrate) of cellulolytic fungi screened from the two fields where again wheat field showed a richer % of cellulolytic fungi (25.76%).

Table 2 : Population dynamics of cellulolytic microflora (g^{-1} dry soil) in different cultivated field.

Location	Fungi (x 10^3)		
	Total*	Cell.**	Cell. %***
Rice Field, Giridhi (Jharkhand)	0.28	0.03	10.35
Wheat Field, Giridhi (Jharkhand)	48.90	12.60	25.76

Total* → Total mycolfora ; Cell.** → Cellulolytic mycoflora ; Cell.%*** → % of Cellulolytic mycoflora w.r.t. total mycoflora

Distribution of cellulolytic fungi in the two crop fields, along with their frequency (%) and relative abundance (%), was projected in Table 3A. Both fields showed the predominance of *Alternaria alternata, Aspergillus fumigatus, Aspergillus niger, Chaetomium globosum, Cladosporium cladosporioides, Coprinus* sp. 1 and 2, *Penicillium funiculosum, Trichoderma lignorum, Trichoderma viride* etc. The occurrence of *Trichoderma* sp. in orchard, woodland and forest soil had also been reported by several mycoecologists (Widen and Abitbol, 1980 ; Roiger *et al.*, 1991). The cellulolytic fungi obtained from lignocellulosic agricultural residues were highlighted in the following Table (3B). While *Aspergillus fumigatus, Aspergillus niger., Chaetomium globosum, Coprinus* sp. 1

Table 3A : Distribution of cellulolytic fungi in cultivated field.

Name of Organism	Frequency (%)	Relative Abundance (%)
Alternaria alternata	60	6.66
Aspergillus fumigatus	60	7.43
Aspergillus japonicus	40	5.65
Aspergillus niger	40	5.65
Aspergillus terreus	30	4.33
Chaetomium globosum	50	6.26
Cladosporium cladosporioides	50	5.18
Coprinus sp. *1*	20	3.36
Coprinus sp. *2*	20	4.30
Drechslera oryzae	30	4.17
Fusarium oxysporum	60	8.15
Myrothecium verrucaria	20	3.78
Penicillium frequentans	60	7.75
Penicillium funiculosum	50	7.25
Rhizopus oryzae	30	3.26
Sporotrichum pulverulentum	40	6.66
Trichoderma lignorum	30	4.26
Trichoderma viride	20	2.72
Trichurus sp.	20	3.18

Table 3B : Distribution of cellulolytic fungi in lignocellulosic agricultural residue

Name of Organism	Frequency (%)	Relative Abundance (%)
Alternaria tenuis	50	7.10
Aspergillus fumigatus	40	4.00
Aspergillus japonicus	50	8.55
Aspergillus niger	40	6.66
Aspergillus terreus	20	2.16
Chaetomium globosum	30	3.26
Coprinus sp. *1*	30	3.33
Coprinus sp. *2*	20	2.25
Drechslera oryzae	30	3.11
Fomes nebulosa	20	2.78
Fusarium chlamydosporum	50	7.20
Humicola insolens	30	4.66
Myrothecium luteum	30	3.77
Penicillium frequentans	60	9.25
Rhizopus oryzae	30	6.67
Sclerotium rolfsii	20	5.25
Sporotrichum pulverulentum	20	3.95
Trichoderma lignorum	50	5.55
Trichoderma viride	50	6.25
Trichurus sp.	20	4.25

and 2, *Trichoderma viride* etc. were common to both agricultural fields and crop residues, some (*Cladosporium viride* etc. were common to both agricultural fields and crop residues, some

(*Cladosporium cladosporioides, Penicillium funiculosum* etc.) were found only in crop fields and some *(Fomes nebulosa, Sclerotium rolfsii* etc.) only in lignocellulosic wastes.

Among them, two potential fungal species *Aspergillus japonicus* and *Penicillium frequentans* were ultimately selected in our saccharification and biodegradation studies as these two were common in both the agricultural fields and wastes.

Rice and wheat straw were chosen as substrate for biodegradation studies. The chemical profile of these crop residues was presented in Table 4A and 4B. It was observed that rice straw contained lower % of both lipid and lignin and higher % of ash and nitrogen than that of wheat.

Table 4A : Analysis of lignocellulosic wastes (minor cell wall constituents)

Substrates	Ash (%)	Lipid (%)	Carbon (%)	Nitrogen (%)	Phosphorus (%)
Rice Straw	13.70	5.0	33.3	0.86	0.06
Wheat Straw	7.63	10.0	31.5	0.28	0.05

Table 4B : Analysis of lignocellulosic wastes (major cell wall constituents)

Substrates	Holocellulose (%)	α-cellulose (%)	Lignin (%)
Rice Straw	70.50	35.12	8.9
Wheat Straw	70.48	33.74	17.0

Results shown are mean of triplicates.

The process of biodegradation of any cellulosic waste was being carried out by the active participation of a group of organism producing cellulase, the cellulose splitting enzyme. In the multi-enzyme complex of cellulase, 3 basic enzymes, viz. endo-1,4-β-D glucanase (EC. 3.2.1.4), exo-1,4-β-D glucanase (EC. 3.2.1.91), endo-1,4-β-D glucanase (Ec. 3.2.1.21), synergistically attacked crystalline cellulose and convert it to cellobiose and glucose.

Production of extra cellular CMCase, FPase and β-glucosidase by different cellulolytic fungi was recorded in Table 5. We had selected *Aspergillus japonicus* for biodegradation of lignocellulosic crop residues which produced appreciable amount of extra-cellular enzymes, were the β-glucosidase (2.60 IU) was recorded highest to CMCase (1.50 IU) and FPase (0.60 IU). Another efficient cellulase producer was screened from agricultural fields of Giridih, identified as *Penicillium frequentans,* which produced high amount of β-glucosidase (2.35 IU), but a comparatively lower CMCase (0.66 IU) and FPase (0.45 IU) (Table 5). Kundu *et al.* (1988) worked upon *Aspergillus japonicus* on moistened wheat bran and measured β-glucosidase and CMCase (endo-β-1-glucanase). β-glucosidase (0.46 U/mg protein) and CMCase (0.48 U/mg protein) produced were lesser than our results.

The species of *Trichoderma, Aspergillus, Sporotrichum, Fusarium* and *Penicillium* are already well known producers of cellulase. It had also been reported that strains of *Trichoderma viride* (together with *Aspergillus niger)* are at present probably the most important cellulase producers for commercial purposes. The fact is to be noted that in our experiment also, *Trichoderma viride* was the highest producer of CMCase, FPase and β-glucosidase. Sahaffner and Toledo (1991) worked with *Trichoderma reesei,* which expressed good cellulase activity (1-2.0 FP U/ml culture) when xylose was used as sole carbon source instead of CMC. For multiple carbon sources (sorbose and xylose) about 2.0 FP U/ml cellulose was produced. The results are more or less similar to ours tested with *T. lignorum* and *T. viride.* Trivedi and Rao (1979) showed that *Trichoderma viride* was one of the better sources of cellulose solubilizing enzymes, but its production of β-glucosidase was relatively low. The β-glucosidase activity of *Aspergillus fumigatus* was about 15-20 times higher than that of *Trichoderma viride.* Sternberg (1976) worked with *Trichoderma viride,* the best known source of extracellular cellulase, and the results produced were compared with our findings. Gokhale *et al.,* (1988) reported the production of cellulolytic enzymes by *Aspergillus niger.* Gayal and Khandeparkar (1998) had studied the production of cellulase by *Penicillum funiculosum* with filter paper strip, as the sole source of carbon and result obtained was 0.33-0.35 IU at 90 hrs. In our experiment, the value of FPase activity was a bit lower (0.18 IU) than that.

Table 5 : Production of extra cellular FPase, CMC-ase and β-glucosidase by various cellulolytic fungi

Organism	CMCase	FPase	β-glucosidase
Alternaria alternata	0.48	0.21	0.60
Aspergillus. terreus	1.00	0.55	2.20
Aspergillus japonicus	1.50	0.60	2.60
Chaetomium globosum	0.58	0.05	0.26
Cladosporium cladosporioides	0.60	0.56	0.62
Drechslera oryzae	1.50	0.50	0.98
Penicillium funiculosum	0.36	0.18	0.48
Penicillium frequentans	0.66	0.45	2.35
Trichoderma viride	3.60	2.60	4.50
Trichoderma lignorum	3.50	2.20	4.20

Results shown are mean of triplicates.

Enzyme unit was expressed as International Unit, which are μ moles of glucose (CMCase), glucose (FPase) and Para-nitrophenol (β-glucosidase) released ml^{-1} filtrate under assay conditins at pH 4.8, incubation temperature 50°C. Canevascini and Gattlen (1981) while carrying on comparative investigation of various cellulase procedures, highlighted on the biosynthesis of cellulolytic enzymes by a number of fungi recognized as *Trichoderma koningii, Fusarium solani, Trichoderma viride, and Sporotrichum pulverutentum.*

Table 6 : Effect of *Aspergillus japonicus* and *Penicillium frequentans* cellulase on saccharification of NaOH treated lignocellulosic wastes

Incubation period – 72 hrs Incubation temperature – 50°C
Substrate connectration – 5%

Organism employed	Substrate	Sugar yield (mg/ml)	% Saccharification
Aspergillus japonicus	Rice straw	35.00	63.00
Aspergillus japonicus	Wheat straw	31.65	57.00
Penicillium frequentans	Rice straw	29.58	53.24
Penicillium frequenttans	Wheat straw	26.85	48.33

Results shown are mean of triplicates.

The data in Table 6 projected the effect of *Aspergillus japonicus* and *Penicillium frequentans* cellulase on saccharification of NaOH treated rice and wheat straw. Here the production of reducing sugar (mg/ml) was highest upon rice straw (35.00 mg/ml) and % saccharification recorded (63.00%) was also highest for *Aspergillus japonicus.* For the same organism, in case of wheat straw, these were 31.65 mg/ml and 57.00% respectively. Coming to *Penicillium frequentans,* % saccharification for rice straw was 53.24% and for wheat straw it was 48.33%. Hence we can say that *Aspergillus japonicus* was a more efficient cellulase producer than *Penicillium frequentans.*

Toyama and Ogawa (1977) reported that delignified sawdust was saccharified efficiently by *Trichoderma viride* cellulase. Shewale and Sadana (1979) showed that enzymatic saccharification was accelerated when rice straw and bagasse were pretreated with alkali.

Van Wyk and Mogale (2000) worked on increased saccharification of pretreated used paper materials by *Trichoderma reesei* and *Aspergillus niger* cellulase mixtures and catalytic profiles of the individual enzymes. They concluded that *Trichoderma reesei* cellulase was more active than *Aspergillus niger.* Pretreatment also proved to be an effective way of enhancing the bioconversion of organic waste as was the case for mixing of cellulases from different microorganisms.

Table 7 : Biodegradation of wheat straw and rice straw by two cellulolytic fungi

Organism employed	Source	% of Biomass degradation	% of Lignin degradation	% of α-cellulose degradation
Aspergillus japonicus	Rice straw	16.66 ± 1.80	15.25 ± 0.75	30.15 ± 1.10
Aspergillus japonicus	Wheat straw	12.50 ± 1.90	11.00 ± 0.68	28.70 ± 9.60
Penicillium frequentans	Rice straw	14.86 ± 1.25	11.73 ± 0.80	27.85 ± 2.50
Penicillium frequentans	Wheat straw	10.60 ± 1.52	8.33 ± 0.48	25.80 ± 4.50

The biodegradation of rice and wheat straw by *Aspergillus japonicus* and *Penicillium frequentans* was represented in Table 7 which measured the amount of biomass, lignin and α-cellulose degradation in case of both the crop residues. It was well evident from the Table that *Aspergillus japonicus* had high lignocellulose degrading capability (16.66% in case of rice straw) and *Penicillium frequentans* was more or less at per with it. Mandhulika *et al.* (1993) also reported different groups of fungi as efficient lignocellulose degraders, especially *Aspergillus, Paecilomyces* and *Sporotrichum.* Antai and Crawford (1981) worked on degradation of softwood, hardwood and grass lignocelluloses by two lignin decomposing *Strepotomyces* strains, namely *S. viridosporus* and *S. setonii.* They concluded that grass lignocelluloses were the preferred substrates for such bioconversions when these two strains were utilized. *S.*

viridosporus and *S. setonii* degraded about 44% and 39%, respectively, of the initial lignin from grass lignocellulose after 12 weeks of incubation. In our case, amount of lignin depletion is 11% for *Aspergillus japonicus* and 8.33% for *Penicillium frequentans* of wheat straw and 15.22% and 11.73% respectively of rice straw after 2 weeks of incubation.

Since cellulose is the world's most abundant biopolymer, it is not surprising that cellulolysis occurs widely in diverse fungal classes. Cellulolysis is normally considered from an ecologica perspective, industrial application or with regard as utilization of celulose for growth (Goyal *et al.*, 1991). Our investigation reports the occurrence of a number of cellulolytic fungi, which produce appreciable amount of cellulolytic enzymes. Cellulolytic enzymes are extensively used in biodegradation and saccharification of cellulose to simple sugar. The recovered sugar may be used as a substrate for fermentation medium for the production of important metabolites like antibiotic, acid, enzymes and single cell protein through biotechnology.

REFERENCES

Black, C. A. (1965). *Methods of Soil Analysis.* Part I. Physical and Mineralogical Properties including Statistics of Measurements and Sampling No. 9 in the Series of Agronomy. American Society of Agronomy, Inc. Madison, Wisconsin, USA.

Canevascini, G. and Gattlen, C. (1981). A comparative investigation of various cellulase assay procedures. *Biotechnol. and Bioeng.* **23** : 1573-1590.

Clarke, D. C. and Christensen, M. (1981). The soil microfungal community of a South Dakota grassland. *Canad J. Bot.* **59** : 1950-1960.

Curl, E. A. and Truelove, B. (1985). *The Rhizosphere.* Springer–Verlag, Berlin.

FAO (1985) *Production Year Book,* Food and Agricultural Organization, Rome, Italy, 39.

Gayal, S. G. and Khandeparkar (1988). Production of cellulase by *Penicillium frequentans. Ind. J. of Microbiol.* **38** : 167-168.

Gilman, J. C. (1975). *A Manual of Soil Fungi* (second Indian reprint). Oxford and IBH Publishing Co., New Delhi. pp. 450.

Goyal, A. ; Ghosh, B. and Eveleigh, D. (1991). Characteristics of fungal cellulases. *Bioresource Technol.* **36** : 37-50.

Jackson, M. L. (1973). In *Soil Chemical Analysis.* 2nd Edition. Prentice Hall of India Private Ltd. New Delhi. pp. 375.

Kundu, K. R. ; Dube, S. and Dubey, K. D. (1988). Extracellular cellulolytic enzyme system of *Aspergillus japonicus. Enzyme and Microbioal Technology,* **10** : 100-109.

Mandhulika ; Singh, D. P. and Malik R. K. (1993). Isolation of a few lignocellulose degrading fungi. *Ind. J. of Microbiol.* **33**(4) : 265-267.

Roiger, D. J. ; Jeffers, S. N. and Cadwell, R. W. (1991). Occurrence of *Trichoderma species* in apple orchards and woodland soils. *Soil Biol. and Biochem.* **23**(4) : 353-359.

Schaffner, D. W. and Toledo, R. T. (1991). Cellulase production by *Trichoderma reesei* when cultured on Xylose based media supplemented with sorbose. *Biotechnol. and Bioeng.* **37** : 12-16.

Shewale, J. G. and Sadana, J. C. (1979). Enzymatic hydrolysis of cellulosic materials by *Sclerotium rolfsii* culture filtrate for sugar production. *Candian Journal of Microbiology,* **25** : 773-783.

Sternberg D. (1976). Beta-Glucosidase of *Trichoderma* : Its biosynthesis and role in saccharification of cellulose. *Applied and Environmental Microbiol.* **31** : 648-654.

Technical Association of Pulp and Paper Industries (TAPPI) (1971). Standard and Suggested Methods, Georgia, USA.

Toyama, N. and Ogawa, K. (1977). In *Bioconversion of cellulosic substrates into energy, chemicals and microbial protein.* Ghose, T. K. (ed.) New Delhi, India.

Trivedi L. S. and Rao K. K. (1979). Production of cellulolytic enzymes by *Aspergillus fumigatus. Indian Journal of Experimental Biology,* **17** : 671-674.

Van Wyk Jacobus P. H. and Mogale Alfred M. (2000). Increased saccharification of pretreated used paper materials by *Trichoderma reesei* and *Aspergillus niger* cellulase mixtures and catalytic profiles of the individual enzymes. *Resource and Environm. Biotechnol.,* **3** : 87-95.

Waksman, S. A. (1961). *Soil Microbiology,* John Wiley and Sons. Inc. New York.

Walker, L. P. and Wilson, D. B. (1991) Enzymatic hydrolysis of cellulose : An Overview. *Bioresource Technol.* **36** : 3-14.

Widen, P. and Abitbal, J. S. (1980). Seasonability of *Trichoderma* species in a Spruce forest soil. *Mycologia,* **72** : 775-784.

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