African Journal of Biotechnology Vol. 7 (11), pp. 1978-1982, 17 June, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Isolation, identification and screening of potential xylanolytic enzyme from litter degrading fungi

Muthusamy Palaniswamy[1]*, Bhathini Vaikuntavasan Pradeep[1] Ramaswamy Sathya[1] and Jeyaraman Angayarkanni[2]

[1]Department of Microbiology, Karpagam Arts and Science College, Coimbatore -641 021, Tamilnadu, India.
[2]Department of Biotechnology, Bharathiar University, Coimbatore – 641 046, Tamilnadu, India.

Accepted 28 July, 2006

Consortia of litter degrading fungal species were developed from different baiting substrates collected in and around Western ghat forest ecosystem, Coimbatore, Tamilnadu, India. Fifty-three litter degrading fungal species were isolated by nylon litterbag technique. The production of endo-β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8), β-D-xylosidase (1,4-β-xylan xylanohydrolase, E.C. 3.2.1.37) and protease was studied using oat spelt xylan as carbon source. Results showed that all fifty-three fungal species isolated from various litter samples produced fairly good xylanolytic enzyme activity. The xylanase and β-D-xylosidase activity ranges from 4.41 to 132.20 U and 48.72 to 1510.32 U, respectively. Growth was determined in terms of mycelial dry weight, which ranged between 0.209 and 1.047 mg/ml. The protease enzyme activity was from 19.7 to 60.8 U. This is the first report concerning xylanolytic enzyme production by the litter degrading fungi, isolated from litter samples.

Key words: Litter degrading fungi, xylanolytic enzyme, xylanase, β-xylosidase.

INTRODUCTION

Agro-industrial and food-processing wastes are available in staggering quantities all over the world, which largely become a source of health hazard. The majority of these wastes contain cellulose (30-40%), hemicellulose (xylan 20-40%), and lignin (20-30%) (Deschamps and Huet, 1985). The utilization of these wastes for the production of strategic chemicals and fuel requires hydrolysis of all the components. Because xylan is a major plant structural polymer, xylanases and the microorganisms that elaborate them could be used in food processing and paper and the pulp, sugar, ethanol, feed, and agrofiber industries (Gomes et al., 1993). For most bioconversion processes, xylan must first be converted to xylose or xylooligosaccharides. They may be achieved by acid hydrolysis or through the use of xylanolytic enzymes. The xylanolytic enzymes include endo-β-1,4-xylanase (1,4--β-D-xylan xylanohydrolase, E.C. 3.2.1.8), β-D-xylosidase (1,4-β-xylan xylanohydrolase, E.C. 3.2.1.37), and de-branching enzymes (esterases) (Chen et al., 1997).

Many microorganisms are known to produce different types of xylanases; the nature of these enzymes varies between different organisms. Many bacterial and fungal species produce the full complement of enzymes necessary to enable them to utilize xylan as carbon source (Hann and Zyl, 2003). Production of both these enzymes has already been reported in a number of micro-organisms, including bacteria (Rani and Krishna Nand, 1996), fungus (Sunna and Antranikian, 1997; Jorgensen et al., 2005), yeasts (Leathers, 1986) and actinomycetes (Nascimento et al., 2003). In the fungal kingdom, a majority of both xylanase and β-D-xylosidase enzymes producing organisms belong to the genus *Aspergillus*, many of which have been well characterized (Ghosh et al., 1993). Filamentous fungi are interesting procedures of this enzyme from an industrial point of view due to extracellular release of xylanases, higher yield compared to bacteria and yeast and production of several auxiliary enzymes that are necessary for debranching of the substituted xylanase (Haltrith et al., 1996). The attention on the applications of xylan degrading enzymes has led to discover many new enzymes with novel characteristics from various microorganisms (Wang et al., 2003).

In the present investigation, we have isolated fifty-three fungal species of various genera [*Acremonium* (2); *As-*

*Corresponding author. E-mail: palaniswamy_m@yahoo.com.

pergillus (17); *Basidiomycetes* (11); *Fusarium* (3); *Humicola* (1); *Mucor* (1); *Penicillium* (13); *Rhizopus* (1) and *Trichoderma* (4)] from Western ghat forest ecosystem by litterbag technique. The fungus produced high titres of endo-β-1,4-xylanase (E.C. 3.2.1.8) and β-D-xylosidase (E.C. 3.2.1.37) extracellulary in shake flask batch cultivation.

MATERIALS AND METHODS

Study site

Mycoflora were isolated from the Siruvani forest of the Western ghat, Coimbatore, Tamilnadu, India (April 2004 – March 2005). The Siruvani forest is located at the foothills of Western ghat extension at 76° 73'N and 10° 58'E and above 500 M a.s.l. The climate in this area is typically monsoonic, characterized by three seasons, viz., warm and moist rainy season (July-October), cold winter season (November-February) and dry and hot summer season (March-June).

Isolation of mycoflora

Litter mycoflora were isolated by nylon net litterbag technique (Palaniswamy, 1997). Paddy straw, Coir pith, *Eucalyptus globulus* and *Tectona grandis* leaves were air dried and used as baits. Each of the air dried material (10 g) was placed in a nylon net bag (1 mm mesh; 20 x 20 cm size) with colored beads (to identify the materials) and buried in the aerable forest soil at 10 cm depth. For each sample, six replicates were maintained. Samples were collected at regular monthly intervals in presterilised polyethylene bags. The litter samples were powdered and suspended in sterile distilled water (0.1 g/50 ml). This suspension (1.0 ml) was plated onto the Petri plate containing rose-bengal-streptomycin-agar medium.

All fifty-three fungal species were identified and deposited at the Research and Development Culture Collection Center, Karpagam Arts and Science College. The fungal species were sub cultured on rose-bengal-streptomycin-agar slants and maintained in a refrigerated condition. The fungal species were identified by morphological and physiological analysis.

Preparation of spore inocula

Conidial spores were harvested from 7 to 10 day old agar slants by suspending spores in water and filtering through gauze into Erlenmeyer flask.

Fermentation

One percent spore suspension (10^6 spores/ml) were inoculated into 250 ml Erlenmeyer flask containing 50 ml of minimal salt medium (Carter and Bull, 1969) composed (g/l) urea 1.4, magnesium sulphate 0.25, calcium chloride 0.05, zinc sulphate 0.02, manganese sulphate 0.02, copper sulphate 0.005, ferrous sulphate 0.1, sodium sulphate 1.0, ethylene diamine tetra acetate 0.6, sodium dihydrogen orthophosphate 1.56, peptone 7.5, yeast extract 2.5, glucose 5.0 and xylan 10.0. Flasks were incubated for 7 days at 30°C in an orbitory shaker at 150 rpm. After the incubation period, the cultures were filtered over a Whatman No.1 filter paper. The residue was used for growth determination. The filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and the clear supernatant was used as enzyme source.

Biomass

Mycelial biomass was collected on a pre-weighed Whatman filter paper, dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

Soluble protein

Soluble proteins were analyzed according to the method of Lowry et al. (1951) after preliminary precipitation with three volumes of 10% TCA. Bovine serum albumin was used as standard.

Mycelial protein

The mycelial protein content was measured in the dry mycelium by Lowry et al. (1951) method as described by Philips and Gordon, 1989.

Xylanase

Endo-β-1,4-xylanase (E.C. 3.2.1.8) activity was measured by DNS method (Miller, 1959). The reaction mixture containing 0.5 ml of 1% oat spelt xylan prepared in 0.05 M sodium citrate buffer pH (5.0), and 0.5 ml of appropriately diluted enzyme were incubated at 50°C for 30 min. One unit (U) was defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute under the above assays conditions (Avcioglu et al., 2005).

β-Xylosidase

β-1,4 xylosidase (E.C. 3.2.1.37) activity was assayed at 50°C using p-nitrophenyl-β-D-xylopyranoside as substrate. p-Nitrophenol liberated was monitored at 430 nm. One unit (U) of enzyme activity was defined as the amount of enzyme, which produced 1 μmole p-nitrophenyl β-D-xylopyranoside per min (Chen et al., 1997).

RESULTS AND DISCUSSION

We adopted litterbag technique for developing promising xylanolytic fungal consortia using the various litter samples, Paddy straw, Coir pith, *Tectona grandis* and *Eucalyptus globulus* collected in and around study area described in materials and methods. In order to select new potential fungal species, fifty-three fungal species were screened for the production of both xylanase (E.C. 3.2.1.8), β-D-xylosidase (E.C. 3.2.1.37) and protease activity when grown by shake flask batch cultivation on oat spelt xylan as the carbon sources. The isolates were screened by measuring xylanolytic and proteolytic activities in shake flask fermentation method. The objective of the present work was to produce a high level of xylanases and less amount of protease activity from xylanolytic fungal consortia. All the fungi exhibited fairly good enzyme activities, with production ranging from 4.41 to 132.20 U.

Table 1 shows the profile of xylanase, β-D-xylosidase and protease production by the fifty-three fungal species. Although fungal growth was abundant on all species

Table 1. Screening of litter degrading fungi for xylanolytic enzyme production

Name of the fungal species	Endo-xylanase activity (IU/ml)	β - D xylosidase activity (IU/ml)	Protease activity (IU/ml)	Soluble protein content (mg/ml)	Fungal biomass (mg/ml)	Mycelial protein (mg/g)
Acremonium furcatum	33.08	73.08	47.8	5.60	0.484	4.0
Acremonium murorum	28.67	48.72	55.7	4.80	0.421	5.2
Aspergillus alutaceus	103.67	276.08	27.8	6.90	0.497	4.2
Aspergillus erythrocephalus	15.44	133.98	28.7	7.38	0.633	3.4
Aspergillus flavipes	90.43	117.74	43.3	7.50	1.047	10.8
Aspergillus flavus	77.20	89.32	37.7	7.39	0.947	5.4
Aspergillus glaucus	79.40	276.08	19.7	3.00	0.209	3.4
Aspergillus japonicus	92.64	85.26	33.8	14.19	0.724	11.6
Aspergillus melleus	50.73	97.44	24.7	5.70	0.425	4.4
Aspergillus nidulans	103.67	97.44	47.8	5.00	0.534	6.4
Aspergillus niger	44.11	64.96	38.3	12.70	0.718	8.0
Aspergillus ornatus	4.41	150.22	30.9	4.70	0.502	7.6
Aspergillus oryzae	26.46	121.80	60.8	5.30	0.525	8.8
Aspergillus sclerotiorum	26.46	251.72	39.4	3.50	0.455	5.0
Aspergillus sydowii	66.17	194.88	38.8	12.30	1.621	12.4
Aspergillus terreus	79.4	1583.40	26.2	4.70	0.372	10.4
Aspergillus ustus	44.11	105.56	40.5	7.50	0.594	6.0
Aspergillus versicolor	44.11	710.51	52.9	4.70	0.416	4.8
Aspergillus wentii	17.64	138.04	32.1	3.19	0.280	3.0
Aspergillus sp.	61.76	113.68	50.6	4.80	0.486	7.6
Basidiomycetes sp. – 1	15.44	308.56	52.4	5.00	0.222	4.0
Basidiomycetes sp. – 2	22.05	600.08	50.6	2.79	0.345	10.2
Basidiomycetes sp. – 3	44.11	893.21	50.6	3.50	0.302	2.8
Basidiomycetes sp. – 4	26.46	267.96	51.8	2.39	0.399	6.0
Basidiomycetes sp. – 5	44.11	1510.32	39.4	5.70	0.306	7.6
Basidiomycetes sp. – 6	22.05	203.00	46.2	4.79	0.371	4.2
Basidiomycetes sp. – 7	63.96	73.08	47.8	4.00	0.317	5.4
Basidiomycetes sp. – 8	37.49	219.24	46.7	3.19	0.268	1.6
Basidiomycetes sp. – 9	30.88	77.14	46.1	1.58	0.470	8.8
Basidiomycetes sp. – 10	35.29	109.62	46.7	3.19	0.430	2.4
Basidiomycetes sp. – 11	35.29	487.20	54.6	3.00	0.441	5.6
Fusarium culmorum	57.34	93.38	50.1	5.50	0.130	3.0
Fusarium dimerum	74.99	227.36	27.8	7.50	0.384	10.8
Fusarium merismoides	55.14	178.64	46.2	5.19	0.370	10.8
Humicola insolens	22.05	73.08	47.8	6.39	0.498	6.4
Mucor circinelloides	77.20	146.16	27.9	12.50	0.751	7.0
Penicillium brevicompactum	22.05	186.76	32.1	4.90	0.537	4.8
Penicillium chrysogenum	15.44	227.36	32.8	5.39	0.507	8.8
Penicillium fellutanum	39.70	470.97	36.0	5.19	0.443	4.8
Penicillium frequentans	103.67	377.58	36.0	12.40	0.465	6.6
Penicillium lividum	81.61	170.52	27.7	5.80	0.364	7.2
Penicillium nigricans	8.82	101.50	32.1	7.50	0.607	12.2
Penicillium restrictum	37.49	316.68	37.7	5.00	0.365	11.2
Penicillium sacculum	28.67	60.90	49.5	2.79	0.878	4.4
Penicillium thomii	44.11	219.20	48.4	5.79	0.442	7.6
Rhizopus oryzae	61.76	194.88	44.4	12.10	0.898	9.2
Penicillium canescens	35.29	259.84	52.3	6.18	0.283	6.0
Penicillium citrinum	33.08	584.64	57.4	3.70	0.396	5.8
Penicillium claviforme	37.49	142.10	33.2	4.80	0.637	7.2

Table 1. Contd.

Trichoderma koningii	127.93	706.45	45.0	4.10	0.529	7.6
Trichoderma pseudokoningii	28.67	211.12	28.1	5.59	0.333	2.6
Trichoderma reesei	22.04	166.46	49.0	9.00	0.285	3.0
Trichoderma viride	132.20	186.76	42.8	2.59	0.320	5.8

All the values are mean of three replicates
Standard error is not given.

there were differences in the production of xylanases and β-D-xylosidase enzymes. *Trichoderma viride* and *Basidiomycetes* sp. – 5 produced highest amounts of xylanase (132.20 U) and β-D-xylosidase (1510.32 U), respectively. Fungal growth in terms of mycelial dry weight was in the range of 0.209 to 1.047 mg/ml. This observation is consistent with the report for *Aspergillus sydowii* MG49 (Ghosh et al., 1993), *Thermomyces lanuginosus* DSM10635 (Xiong et al., 2004) and *Aspergillus fumigatus* AK1 (Anthony et al., 2003). Production of extracellular soluble protein was high in all the test fungus. Extracellular soluble protein concentration ranged between 3.0 and 14.19 mg/ml. The same phenomenon was described for *Trichoderma reesei* M-7 (Jamas et al., 2002), *Aspergillus sydowii* MG49 (Ghosh et al., 1993), *Streptomyces* sp. (Georis et al., 2000) and *Streptomyces* sp. strain AMT-3 isolated from Brazilarin cerrado soil (Nascimento et al., 2003).

In addition to xylanase, the organisms produced protease enzyme. The presence of protease may affect the stability of xylanase during extraction and storage (Gessesse and Mamo, 1999). Therefore, the use of appropriate protease inhibitors during extraction or selection of protease-deficient mutant may solve the effect of protease. Agosin et al. (1987) have shown that the protease secreted by the white rot fungus *Dichomitus squalens* was responsible for the decrease in xylanase activity. Cultures of *Aspergillus awamori* and *Aspergillus phoenicis* was found to contain a protease which was capable of inactivating the xylanase enzymes even at 4°C (Smith et al., 1991). It was therefore considered essential that the protease titers of the selected organisms be as low as possible. Protease enzyme activity was maximum for *Aspergillus oryzae* (60.8 U) and low for *Aspergillus glaucus* (19.7 U). Similar findings were reported with *Bacillus* sp. (Gessesse and Mamo, 1999; Balakrishnan et al., 1997; Avcioglu et al., 2005) and *Pichia stipitis* (Srikrishna et al., 1997). The results were also in good agreement with production of xylanase enzyme obtained from various bacteria, actinomycetes, yeast, fungi which was studied extensively by many workers (Milagres et al., 1993; Rani and Nand, 1996; Beg et al., 2000; Passoth and Hahn-Hageradl, 2000; Wang et al., 2003; Nacimento et al., 2003; Lama et al., 2004; Avcioglu et al., 2005; Jorgensen et al., 2005; Shah and Madammar, 2005).

In conclusion, this is the first report of the production of xylanolytic and proteolytic enzyme by litter degrading fungi using shake flask batch culture method. Further studies on optimization of xylanase production by using natural lignocellulosic substrates, evaluation of Industrially important thermostable xylanase, neutral xylanase enzyme for food processing industries and using various techniques to produce low titre or free of proteolytic enzyme activity is in progress.

ACKNOWLEDGEMENTS

This work was financially supported by the University Grants Commission (UGC), Government of India, New Delhi (Grant no. F-3-41/2004 (SR)). The author (MP) express his gratitude to the Management, Karpagam Arts and Science College, Coimbatore, Tamilnadu, India for providing facilitates and encouragement.

REFERENCES

Agosin E, Rouau X, Brillouet JM (1987). Fermentation of wheat straw by the white rot fungus *Dichomitus squalens*. Can. J. Microbiol. 33: 1050-1054.

Anthony T, ChanadraRaj K, Rajendran A, Gunasekaran P (2003). High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. Enzyme Microbiol. Technol. 32: 647-654.

Avcioglu B, Eyupoglu B, Bakir U (2005). Production and characterization of xylanases of a *Bacillus* strain isolated from soil. World J. Microbiol. Biotechnol. 21: 65-68.

Balakrishnan H, Srinivasan MC, Rele MV (1997). Extracellular protease activities in relation to xylanase secretion in an alkalonphilic *Bacillus* sp. Biotechnol. Lett. 19: 599-601.

Beg QK, Bhushan B, Kapoor M, Hoondal GS (2000). Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. Enzyme Microbiol. Technol. 27: 459-466.

Carter BLA, Bull AT (1969). Studies of fungal growth and intermediary metabolism under steady and non-steady conditions. Biotechnol. Bioeng.11: 785-804.

Chen C, Chen JL, Lin TY (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. Enzyme. Microbiol. Technol. 21: 91-96.

Deschamps F, Huet MC (1985). Xylanase production in solid-state fermentation: a study of its properties. Appl. Microbiol. Biotechnol. 22: 177-180.

Georis J, Giannotta F, Buyl ED, Granier B, Frere JM (2000). Purification and properties of three endo-β-1,4- xylanases produced by *Streptomyces* sp strain S38 which differ in their ability to enhance the bleching of kraft pulps. Enzyme Microbiol. Technol. 26: 178-186.

Gessesse A, Mamo G (1999). High-level xylanases production by an alkaliphilic *Bacillus* sp by using solid state fermentation. Enzyme Mirobiol. Technol. 25: 68-72.

Ghosh M, Das A, Mishra AK, Nanda G (1993). *Aspergillus sydowii* MG

49 is a strong produce of thermostable xylanolytic enzymes. Enzyme Microbiol. Technol. 15: 703-709.
Gomes J, Purkarthiofer HM, Kapplomiller J, Sinnner M, Steiner W (1993). Production of high level of cellulase–free xylanase by the thermophilic fungus, *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellolosic materials. Appl. Microbiol. Biotechnol. 39: 700-707.
Haltrich D, Nidetzky B, Kulde KD, Steiner W, Zupancic S (1996). Production of fungal xylanases. Biores. Technol., 58: 137-161.
Hann RD, Van Zyl WH (2003). Enhanced xylan degradation and utilization by *Pichia stipitis* over producing fungal xylanolytic enzymes. Enzyme. Microbiol. Technol. 33: 620-628.
James P, Targonski Z, Mleko S (2002). The effect of xylose, galactose and sorbose on the production of selected extracellular enzymes by mutant strain Trichoderma reesei M-7 during continuous cultivation. Biotechnologia. 1: 195-207.
Jorgensen H, Makeberg A, Krogh KBR, Olsson L (2005). Production of celluases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulose adsorption by capillary electrophoresis. Enzyme Microbiol. Technol. 36: 42-48.
Lama L, Calaudrsilli V, Gambacorta A, Nicolaus B (2004). Purification and chrecterization of thermostable xylanase and -β-xylosidase by the thermophilic bacterium *Bacillus therantarctcus*. Res. Microbiol. 155: 283-289.
Leathers TD (1986). Color variants of *Aureobasidum pullulans* overproduce xylanase with extremely high specific activity. Appl. Environ. Microbiol. 52: 1026-1030.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
Milagres AMF, Lacis LS, Prade RA (1993). Characterization of xylanase production by a local isolate of *Penicillium janthinellum*. Enzyme Microbiol. Technol. 15: 248-253.
Miller GL (1959). Use of dinitro salisilic acid reagent for determination of reducing sugar. Ann. Chem. 31: 426-428.
Nascimento RP, Marques S, Alves L, Girio F, Amaral–Collalo MT, Sacramento DR, Silva EP, Reed BR, Coelho R (2003). A novel strain of S*treptomyces malayriensis* isolated from Brazilian soil produces high endo -β-1,4-xylanase titres. World J. Microbiol. Biotech. 19: 879-881.
Palaniswamy M (1997). Exploitation of litter degrading fungi for ethanol production and biobleaching of hardwood Kraft pulp, Ph.D. thesis, Bharathiar University, Coimbatore, India.
Passoth V, Halm-Hagerdal B (2000). Production of a heterologous endo1,4-β-xylanase in the yeast *Pichia stipitis* with an O_2 regulated promoter. Enzyme Microbiol. Technol. 26: 781-784.
Philips MW, Gordon GLR (1989). Growth characteristics of an cellobiose of three different anaerobic fungi. Appl. Environ. Microbiol. 55: 1695-1702.
Rani S, Nand K (1996). Development of cellulase free xylanase producing anaerobic consortia for the use of lignocellulosic waste. Enzyme Microbiol. Technol. 18: 23-28.
Shah AR, Madamwar D (2005). Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem. 40: 1763-1771.
Smith DC, Wood TM (1991). Xylanase production by *Aspergillus awamori* development of a medium and optimisation of extracellular xylanase and β-xylosidases while maintaining low protease production, Biotechnol. Bioeng. 38: 883-890.
Srikrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsag JT, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA (1997). Strategies for optimal synthesis and secretion of heterologus proteins in the methylotrophic yeast *Pichia pastrsis.* Gene, 190: 55-62.
Sunna A, Antranikian G (1997). Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. 17: 39-67.
Wang SL, Yen YH, Shih IL, Chang AC, Chang WT, Wu WC, Chai YD (2003). Production of xylanases from rice bran by *Streptomyces actuosus* A-151. Enzyme Microbiol. Technol. 33: 917-925.
Xiong H, Nyyssola A, Janis J, Pastnen O, Weymarn NV, Leisola M, Turnman O (2004). Characterization of xylanase produced by submerged cultivation of Themomyces langinosum DSM 10635. Enzyme Mirobiol. Technol. 35: 93-99.