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Full Length Research Paper

Isolation, identification and screening of potential cellulase-free xylanase producing fungi

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In order to isolate cellulase-free xylanase-producing fungi, screening and isolation of fungi was done using decaying wood, agricultural wastes and other lignocellulosic wastes as microbial source. Thirty (30) fungal species were selected for further analysis based upon clearing zones formation on xylan enriched agar plates. Submerged fermentation was done with 30 fungal isolates to identify strain that could produce highest amount of cellulase-free xylanase at a pH of 5.5 and at 28 ± 2°C temperature. Under screening conditions, 90% of the strains produced xylanase (6.6 to 495 U/ml) and negligible amount of cellulase (≤ 0.6 U/ml) with growth. Growth was determined in terms of mycelial dry weight which ranged between 0.6 to 2.34 mg/ml. The amount of soluble protein was also determined which ranged between 102 to 300 µg/ml. The pH change after incubation was in between 5.0 to 8.0. Strain specific variability in xylanase production was confirmed in *Aspergillus* sp. and *Penicillium* sp. In addition, this study shows here that *Rhizopus* sp. can also produce xylanase under given conditions.

Key words: Cellulase-free xylanase, xylan, xylanase, submerged fermentation, malt extract agar.

INTRODUCTION

Paper and pulp industry is one of the major sources of pollution, generating large volumes of intensely colored effluent for each metric ton of paper produced (Ali and Sreekrishnan, 2001). In the paper production process, pulping is a step where cellulose fibers are broken apart and lignin is removed by using chlorine. This step even though necessary, is the prime cause of pollution as elemental chlorine reacts with lignin to form chlorinated lignin derivatives such as chlorolignols, dioxins and sulfur compounds. In addition, other organic matter in the pulp reacts with chlorine to form adsorbable organic halides which are carcinogenic, toxic and recalcitrant to degradation (Ali and Sreekrishnan, 2001). Owing to this problem and strict governmental regulation, more companies are investigating alternative methods such as biobleaching, hydrogen peroxide or oxygen based delignification.

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Biobleaching is a process where xylanolytic and/or lignolytic enzymes, instead of chlorine, are used to break the xylan, cellulose, lignin and other compounds during pulping process. Currently, biobleaching is carried out using white-rot fungi derived lignolytic enzymes (e.g manganese peroxidase, laccase) or by using hemicellulolytic enzymes (Casimir et al., 1996). Recently, xylanolytic enzymes are receiving increasing attention because of their potential applications in improving digestibility of animal feed, bioconversion of lingocelluloses into feed-stocks, fuels and in pre-treatment of pulps prior to bleaching in pulp and paper industry (Kim et al., 2000; Rifaat et al., 2005).

Xylanolytic enzymes catalyze the hydrolysis of xylan, the major constituent of hemicellulose, which is the second abundant molecule in plant cell wall (Coughlan and Hazlewood, 1993). Xylan is a branched heteropolysaccharide constituting a backbone of β-1, 4 linked xylopyranosyl units substituted with arabinosyl, glucuronyl and acetyl residues (Shallom and Shoham, 2003). Biodegradation of xylan is a complex process that requires the coordination of several xylanolytic enzymes

which hydrolyze xylan and arabinoxylan polymers. This enzyme group includes endo-β1,4-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), which attack the main chain of xylans and β-D-xylosidase (1,4-β-xylan xylanohydrolase, EC 3.2.1.37), which hydrolyze xylo-oligosaccharides into D-xylose, in addition to a variety of debranching enzymes that is, α-L-arabinofuranosidases, α-glucuronidases and acetyl esterases (Collins et al., 2005). Many of the xylanase producing microorganisms express multiple isoforms that have been ascribed to a variety of reasons that is, heterogeneity and complexity of xylan structure (Kormelink and Voragen, 1993). Literature review shows that, a large variety of microorganisms, including bacteria, yeast and filamentous fungi have been reported to produce xylanase (Wong et al., 1988). From an industrial perspective, fungi are interesting due to higher extracellular release of xylanases, in addition to production of several auxiliary enzymes that are necessary for debranching of the substituted xylans (Haltrich et al., 1996). Therefore, the main objectives of the study were isolation of potential xylanase producing fungi, but not cellulase, from environmentally exposed agricultural soils, wood-wastes and other lignocellulose wastes and to identify strains that secrete maximum amount of cellulase-free xylanase during submerged fermentation. In the present investigation, thirty fungal strains of various genera (*Alternaria, Aspergillus, Curvularia, Drechslera, Fusarium, Penicillium, Rhizopus, Trichoderma*, etc) were isolated and screened for xylanase activity.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used were of analytical grade. Bovine serum albumin, malt extract agar medium, potato dextrose agar and carboxy methyl cellulose were obtained from Hi-media, Mumbai, India. Birch wood xylan was purchased from Sigma chemicals Co., USA.

Isolation and screening of fungi

Samples were collected from various hemicellulose containing substrates (decaying wood, agricultural wastes, agricultural soils and lignocellulosic wastes) which were exposed to the atmosphere and suspended in sterile distilled water. Suspensions after serial dilution were spread on potato dextrose agar (PDA) medium containing (g/L): potato infusion, 200; dextrose, 20 and agar, 15. The fungal isolates were sub-cultured to purity and were preserved on potato dextrose agar slants under refrigeration conditions. Based on cell and colony morphology characteristics, 30 fungal species were isolated and identified, using standard reference manuals (Ellis, 1976; Raper and Fennel, 1965). The fungal isolates were preserved on PDA medium on slants or plates, whereas, all the experiments were done either in malt extract or Mandels and Sternburg's basal medium supplemented with either xylan or cellulose.

Screening for xylanolytic activity on malt extract agar medium

Thirty (30) fungal isolates were screened by xylan-agar diffusion method for their abilities to produce extracellular xylanase during their growth on enriched malt extract agar medium (MEA) containing xylan as the sole carbon source (Nakamura et al., 1993). The composition of the medium was (g/l): birch wood xylan, 1.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 0.2 and agar 20.0. The inoculated plates were incubated for 5 days at 28 ± 2°C. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis after flooding the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl (Teather and Wood, 1982). Fungal strains, which produced distinct clearing zones around their colonies, were selected. The amount of xylanase produced was quantified under submerged fermentation condition.

Xylanase production under submerged fermentation

For xylanase production under submerged conditions, Mandels and Sternburg's basal medium supplemented with 10% birch wood xylan was used (Mandels and Sternburg, 1976). The Mandel's medium was prepared with the following composition (g/l): urea, 10; peptone, 0.3; yeast extract, 0.75; $(NH_4)_2SO_4$, 0.25; KH_2PO_4, 1.4; $CaCl_2$, 2.0; $MgSO_4.7H_2O$, 0.3; $NaNO_3$, 3.0; KCl, 0.5 and trace elements (mg/l): $CuSO_4.5H_2O$, 0.5; $FeSO_4.7H_2O$, 0.5; $MnSO_4.4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20.0 and Tween-80, 0.1% (v/v) pH 5.5. For the incolulation of 25 ml of culture, 5 discs each of 5.0 mm in diameter were obtained by using a sterile cork borer from PDA culture plate containing fungal lawn. Inoculated flasks were incubated at 28 ± 2°C under static conditions for 7 to 14 days. The culture medium was filtered using Whatmann no.5 filter paper, the filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source.

Quantitative assay for xylanase activity

The amount of xylanase produced was measured by using 1% birch wood xylan as the substrate (Bailey et al., 1992). Xylanase activity was assayed in 3.0 ml of a reaction mixture containing 1.0 ml of crude extracellular enzyme source, 1 ml of 1% birch wood xylan (prepared in 0.05 M Na-citrate buffer, pH 5.3) and 1 ml of 0.05 M citrate buffer. The mixture was incubated at 55°C for 10 min. The reaction was stopped by the addition of 3.0 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 15 min (Miller, 1959). After cooling, the color developed was read at 540 nm. The amount of reducing sugars liberated was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of xylose in 1 min under assay conditions (Khan et al., 1986).

Quantitative assay for cellulase activity

Cellulase (CMCase) activity was determined by mixing 1.0 ml of 1% (w/v) CMC (prepared in 50 mM Na-acetate buffer pH 5.3) with 1.0 ml of crude extracellular enzyme source and incubating at 50°C for 15 min (Casimir et al., 1996). The reaction was stopped by the addition of 3.0 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 15 min. The colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 µmol of glucose equivalents per minute under the assay conditions (Mandels et al., 1981).

Soluble protein assay

Protein content of the culture supernatant was determined according

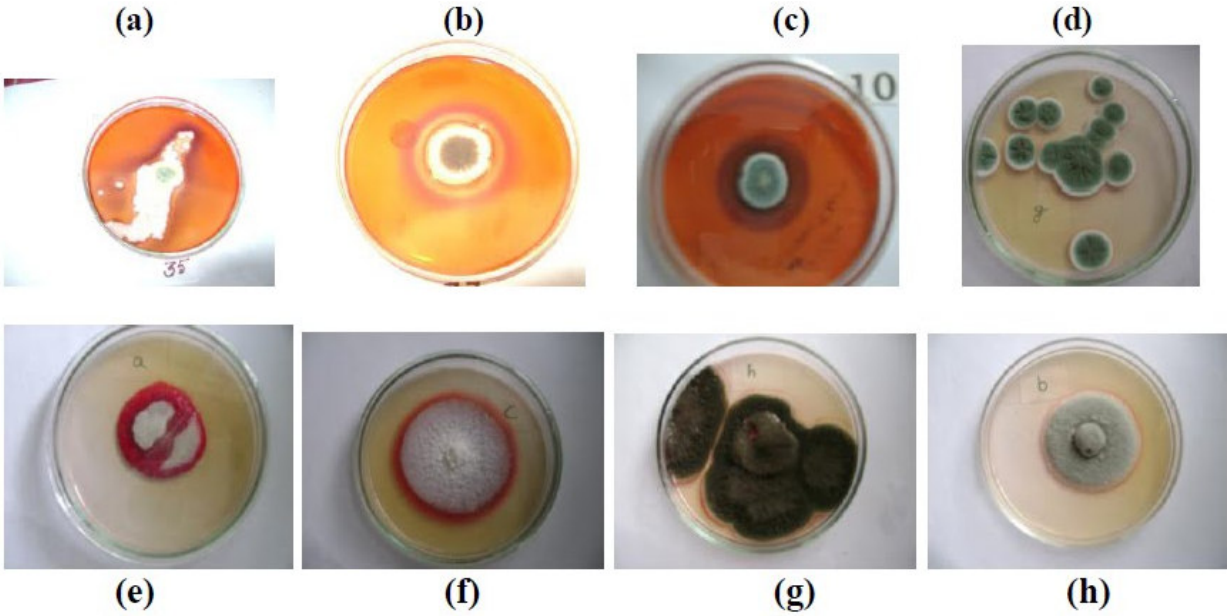

Figure 1. Relative activities of xylanases (hydrolyzed zone in cm) produced by fungi. (a) *Trichoderma CDC-140;* (b) *A. niger CDC-38;* (c) *Penicillium CDC-100;* (d) *Penicillium CDC-94;* (e) *Alternaria CDC-64;* (f) *FusariumCDC-84;* (g) *Rhizoctonia CDC-115;* (h). *Drechslera CDC-80.*

to the method described Lowry et al. (1951) using bovine serum albumin as standard.

Mycelial dry weight

Mycelial dry biomass was collected on a pre-weighed Whatmann filter paper 5, dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

pH determination

Change in pH in culture filtrate after 14 days of incubation was determined using pH paper.

Statistical analysis

To determine significance, linear regression was done using Sigma-Stat under default settings (SigmaStat; SPSS Inc., Chicago, IL).

RESULTS

The objective of present investigation was to isolate fungal strains with high level of xylanases and no or less amount of cellulase producing ability. In order to achieve the aims, we have selected agricultural wastes, decaying wood and lignocellulose wastes as they are rich sources of cellulose (30 to 40%), hemicellulose (xylan 20 to 40%) and lignin (20 to 30%). During the initial screening, a total of 30 different fungal strains were isolated on PDA medium. Identification of fungal strains was made on the basis of morphological, cultural and reproductive characteristics by using standard reference manuals (Ellis, 1976; Raper and Fennel, 1965). Among the 30 isolates, almost 50% belong to *Aspergillus* species, forming a dominant group, followed by *Penicillim* species and *Trichoderma* species in all the above mentioned sources. All the fungi were screened for extracellular xyanase activity on malt extract agar medium containing xylan as sole carbon source (Figure 1).

Xylanases in combination with cellulases have applications in food processing (Biely, 1985) whereas, xylanases without former are important in paper industry as the quality of paper depends upon the amount of cellulose present in the paper. Therefore, to select potential cellulase-free xylanase producing fungi, submerged fermentation was carried out and enzyme activities (xylanase and cellulase) were measured. For all the 30 isolates, both qualitative (zone of inhibition) and quantitative (U/ml) xylanase assays were done (Table 1). From the table it is clear that, all the fungi under study secreted xylanase enzyme at varied levels. The diameter of the hydrolyzed zones ranged from 2.8 cm (*Trichoderma* sp. *CDC-125*) to 7.0 cm (*Drechslera* sp. *CDC-80*). All *Aspergillus* species secreted substantial levels of xylanase enzyme which ranged from 3.4 to 6.9 cm. The four *Penicillium* species showed moderate enzyme activity (5.0 cm). Interestingly, *Trichoderma* species, popular in biological control of plant diseases showed its broad range of xylanase activity where *Trichoderma* sp. *CDC-125* showed 2.8 cm of hydrolytic zone and 150 U/ml under submerged conditions while *Trichoderma* sp. *CDC-140* recorded 5.9 cm and 450 U/ml.

Table 1. The qualitative and quantitative assay of xylanases by thirty fungi isolated from agricultural soil (AGS), lignocellulosic wastes (LCW), wood wastes (WWS).

S/N	Sample I.D	Name of fungal specie	Qualitative assay		Quantitative assay			
			Hydroly-zed zone (cm)	pH	Xylanase Activity (U/ml)	Cellulase activity (U/ml)	Soluble protein content(µg/ml)	Mycelial dry weight (mg)
01	WWS	*A. flavus CDC-1*	5.9	5.0	90.0	0.2	120	0.70
02	AGS	*A. flavus CDC-6*	5.9	5.0	28.5	---	102	0.60
03	LCW	*A. flavus CDC-12*	4.6	7.0	21.0	0.32	240	1.46
04	LCW	*A. flavus CDC-20*	3.8	7.0	14.4	0.6	270	1.26
05	LCW	*A. fumigatus CDC-30*	5.5	7.0	321.0	0.6	170	1.50
06	AGS	*A. glaucus CDC-33*	4.0	5.0	5.1	0.32	200	1.00
07	LCW	*A. niger CDC-38*	5.5	7.0	369.0	---	170	1.90
08	WWS	*A. niger CDC-40*	5.0	5.0	252.0	0.4	250	0.80
09	WWS	*A. ochraceus CDC-41*	6.9	7.0	198.0	0.4	260	1.30
10	LCW	*A. terreus CDC-45*	4.0	7.0	6.6	0.4	120	1.50
11	AGS	*A. parasiticus CDC-48*	5.2	5.5	273.0	---	300	1.30
12	LCW	*Aspergillus* sp *CDC-50*	5.6	5.5	31.5	---	280	2.10
13	WWS	*Aspergillus* sp *CDC-57*	4.0	8.0	28.5	---	170	1.20
14	WWS	*Aspergillus* sp *CDC-60*	3.4	7.0	12.0	0.2	260	1.30
15	LCW	*Alternaria* sp *CDC-64*	5.6	7.0	240.0	0.4	256	1.32
16	LCW	*Curvularia* sp *CDC-71.*	4.0	8.0	45.0	0.2	140	1.93
17	LCW	*Drechslera* sp *CDC-80*	7.0	5.0	210.0	0.1	136	1.36
18	LCW	*Fusarium sp CDC-84*	6.5	6.0	240.0	0.2	164	1.39
19	WWS	*Neurospora sp CDC-88*	3.9	7.0	11.4	0.2	180	1.20
20	AGS	*Penicillium sp CDC-89*	4.2	6.0	12.0	0.2	200	2.000
21	LCW	*Penicillium* sp *CDC-94*	5.0	7.0	228.0	0.1	200	1.85
22	LCW	*Penicillium* sp *CDC-97*	5.2	7.0	255.0	0.1	282	1.80
23	WWS	*Penicillium* sp *CDC-100*	5.0	7.0	264.0	0.1	220	1.80
24	LCW	*Phoma* sp *CDC-111*	5.0	7.0	210.0	0.2	200	2.00
25	LCW	*Rhizoctonia* sp *CDC-115*	5.0	7.0	249.0	---	200	2.34
26	AGS	*Rhizopus* sp *CDC-120*	4.6	7.0	234.0	---	220	1.61
27	LCW	*Trichoderma* sp *CDC-125*	2.8	7.0	150.0	0.2	300	1.20
28	WWS	*Trichoderma* sp *CDC-140*	5.9	5.0	495.0	.2	176	1.88
29	LCW	*Trichothecium* sp *CDC-148*	5.0	7.0	9.0	0.2	164	2.10
30	AGS	*Sterile hyphae CDC-150*	5.0	7.0	31.2	0.2	120	1.60

Overall, the maximum xylanase production was recorded in *Trichoderma* species *CDC-140* (495 U/ml), while minimum was recorded in *Aspergillus glaucus CDC-33* (5.1 U/ml) (Table 1). A lot of diversity in the xylanase production was noticed among 14 isolated *Aspergillus* species, which ranged between 5.1 to 369 U/ml. The phytopathogenic fungi responsible for causing wilt diseases (Maheshwari et al., 1981), *Fusarium* sp. *CDC-84* produced moderate (240 U/ml) range of xylanase. *Neurospora* species *CDC-88,* popular in studies on fungal genetics produced very meager amounts of xylanase enzyme (11.4 U/ml). Out of 4 *Penicillium* sp. isolated*, CDC-89* strain produced very low xylanase (12.0 U/ml), while the remaining three showed moderate activity (250 U/ml). Among the 30 fungal isolates, six species failed to secrete cellulase enzyme, while the other fungi produced very minimum quantities of xylanase enzyme which ranged between 0.1 to 0.6 U/ml. *Aspergillus flavus CDC-20* and *Aspergillus fumigatus CDC-30* were responsible for maximum (0.6 U/ml) secretion of cellulase enzyme. *Trichoderma* species showed moderate (0.2 U/ml) cellulase activity while *Penicillium* species were the meager (0.1 U/ml) enzyme activities.

In microbes, the enzyme production is usually associated with the growth phase (Tlecuitl-Beristain et al., 2008). Therefore, to rule out or confirm the role of growth phase in enzyme production, the mycelia dry weight of all the fungal isolates was determined and results showed that, there was no correlation (R^2 = 0.0524) between the dry weight of the mycelium with the production of xylanase and cellulase enzymes (Table 1). Therefore, we believe that the enzyme production is not depended upon the growth phase of fungi. This observation was similar irrespective of growth conditions that is, solid agar medium or broth. The maximum mycelial dry weight was reported in *Rhizoctonia* species *CDC-115* (2.34 mg), while the lowest was noticed for *Aspergillus* sp (1.0 to 1.5 mg). The pH fluctuations during the 14 days of incubation were very less and showed their range from 5.0 to 8.0. The soluble protein content of the 30 fungi was calculated and the range was between 102 µg/ml (*A. flavus CDC-6*) to 300 µg/ml (*Trichoderma* species *CDC-125*).

DISCUSSION

Even though a large number of bacteria, yeast and filamentous fungi have been reported to produce xylanase (Wong et al., 1988), filamentous fungi are gaining importance as producers of xylanase over others from the industrial point of view due to non pathogenic nature, ease in cultivation under fermentation conditions and capable of producing high levels of extracellular enzymes (Kar et al., 2006). Hence, we decided to focus our attention on the isolation of fungi with xylanase producing ability. Under natural conditions, depending upon various external factors, microflora varies from sample to sample. Therefore, in order to rule out any biased selec-tion criteria, we have selected samples from different agricultural soils, wood-wastes and other lignocellulosic wastes in our initial screening approach. It is noteworthy that, the initial isolation and enumeration of the xylanases producers was not carried by using a substrate specific selective medium, even though, most of the isolates were found positive to the cellulase- free xylanase production. We believe that there could be two possibilities; one natural substrate in the agricultural wastes might have acted as selection for enumeration or allowed the growth of those organism which produce the xylanase enzyme under natural conditions. As our aim was isolation of fungal isolates, in order to avoid the growth of bacteria which overgrows the fungi in very short time, acidic pH have been selected during our screening. Probably due to these factor microbes in symbiotic association with the fungi did not appear in the screening.

During our screening on PDA plates, we obtained 30 isolates belonging to 9 different genera with no correlation among the total counts, number of genera and species isolated from the three different sources (Table 1). One of the major problems in screening large number of microbial strains for their xylanase producing ability, is the lack of single rapid reliable screening technique. Hence, solid agar screening method was used for screening and confirmed the xylanase production under submerged conditions by using the standard procedures. Initially, solid screening medium containing xylan as the sole carbon source developed for this purpose was employed (Flannigan and Gilmour, 1980). Xylanase producing organisms were first identified on the basis of the clearing zone formed around the colonies (Table 1). This technique, although useful, has its limitations. For example, Tseng et al., (2000) have found that, some of the strains previously identified as potential-enzyme producing microbes on solid screening methods, did not produce any enzyme in liquid broth. In contrast, some strains, which were identified as negative, were shown to produce high amounts of enzyme (Teather and Wood, 1982; Tseng et al., 2000). Therefore, in order to rule out the omission of any isolate due to experimental limitation, all the isolates were cultured in liquid culture systems for enzyme production.

It is a routine practice in screening procedures; the isolates which produce higher zone of inhibition or clearance are selected for further studies. However, we believe that this should not be a case for xylanase enzyme as screening in solid or semi-solid based method has some technical problems which can influence clearing zone size. For example, in addition to growth kinetics of the isolates, the migration of enzyme released depends upon the agar percentage, molecular size and weight of the enzyme, and temperature of the growth. Even though culturing the isolates in liquid systems does not pose any severe technical restrictions, it is practically

challenging and does not give an indication about the enzyme productions rates, etc., during the study. Therefore, in order to see whether there is any correlation between the clearing zone and enzyme production, a regression analysis was done. Our results show (data not shown) that there is no correlation (R^2 = 0.242) between the zone of the clearing and the enzyme units produced. This data indicates that, zone of inhibition is not a perfect marker to decide the highest producer, however, might indicate about the selection of isolates (Teather and Wood, 1982; Tseng et al., 2000). Therefore, we argue that, in case of xylanase screening, both the solid based screening as well as the liquid culture confirmation methods has to be done.

This investigation, in accordance with others (Anthony et al., 2003; Ghosh and Nanda, 1994; Hrmova et al., 1991), have showed that the highest xylanase producing strain, with less or no cellulase activity, belong to the genus *Aspergillus* sp. Interestingly, strain specific variability in xylanase production was observed in the *Penicillium* species isolated from agricultural wastes and lignocellulose wastes as reported by others (Haas et al., 1992; Palma et al., 1996). Out of all the isolates, *Fusarium CDC-84,* with high xylanase activity, low cellulase production and biomass, showed promising industrial applicable results. These results are in consistence with the results obtained by Gupta et al. (2009), where they showed the xylanase production ability by *Fusarium* species for the first time. Out of all the isolates, owing to ease in cultivation and efficient secretory ability of xylanase, it is believe that *Fusarium* could be a useful organism for the large-scale production of enzymes for paper and pulp industry (Gupta et al., 2009; Wood and Mc Crae, 1986).

It has been shown by various researchers that xylanase production under submerged cultures is not a true test to confirm the cellulase-free xylanase production as purified xylans can be excellent substrates for xylanase production and are frequently used for small-scale experiments (Haltrich et al., 1996). Therefore, to rule out the production of cellulase enzyme in the presence of substrate, we have grown all xylanase positive isolates on a carboxy methyl cellulose rich medium and on agricultural waste, which is near identical to natural conditions. In comparison to xylanase, results show that cellulase production was negligible (less than 100 folds) or absent in 90% of strains when grown on agricultural wastes or cellulose (CMC) indicating its strict non-cellulolytic nature (results not shown here). We are well aware that irrespective of the medium used in combination with substrate, the conditions such as degree of aeration, pH and temperature during fermentation regulate the enzyme production (Nair et al., 2008). Biswas et al. (1990) and Gilbert et al. (1992) have shown that, even though the screening is carried out on pure and defined substrate, there is every probability to isolate cellulase-free xylanase producing fungi (Biswas et al., 1990; Gilbert et al., 1992). Therefore, it is assumed that many of the isolates are cellulase-free xylanase. Interestingly, even with lack of robust universal screening method, difference in the sample collection locations, time etc our results are comparable to the results obtained by various researchers. An observation noted during the statistical analysis is that, there is no correlation between the enzyme production, mycelium growth and soluble protein in the culture filtrate.

Further studies on optimization of xylanase production by using natural lignocellulosic substrates, purification and characterization of xylanase by potential xylanolytic fungal strains are in progress.

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