Full Length Research Paper

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

Rajashri D. Kamble[1]* and Anandrao R. Jadhav[2]

[1]Department of Biotechnology, Tatyasaheb Kore Institute of Engineering and Technology, Warananagar, Pin- 416113.Tal. Panhala, Dist. Kolhapur, Maharashtra, India.
[2]Department of Microbiology, Smt. K.R.P. Kanya Mahavidyalaya, Islampur, Pin 415 414, Tal: Walwa Dist: Sangli, Maharashtra, India.

In order to isolate cellulase-free xylanase producing fungi, screening and isolation was done using composting soil as microbial source. Eight fungal species were selected for further study based on clearing zones formation on agar media containing covalently linked xylan with dye cibacron brilliant red-3BA. Both solid state and submerged fermentations were done with eight 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 solid state conditions, 50% of the strains produced xylanase (45.78 to 923 U gds^{-1}) and lower amount of cellulase (20 to 33 U gds^{-1}). The amount of soluble protein was also determined which ranged between 67 to 99 mg gds^{-1}. In submerged conditions, 25% strains produced xylanase (45 to 205 U mL^{-1}) and negligible amount of cellulase (12 to 13 U mL^{-1}). The amount of soluble protein was determined which ranged between 45 and 85 U ml^{-1} in submerged conditions. Growth was determined in terms of mycelial dry weight which ranged between 0.70 and 2.90 mg mL^{-1}.

Key words: Cellulase-free xylanase, xylan, solid state fermentation, submerged fermentation.

INTRODUCTION

Xylan being major hemicellulose constituent of wood, is the second most abundant renewable polysaccharide after cellulose. Xylan consist of a homopolymeric backbone of β-1, 4 linked D-xylopyranose and short chains of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues (Kulkarni et al., 1999). Biodegradation of xylan is a complex process that requires the coordination of several xylanolytic enzymes which hydrolyze xylan and arabinoxylan polymers (Biely, 1985). Xylanolytic enzyme group includes endo-β-1,4-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 xylooligosaccharides into D-xylose, in addition to a variety of debranching enzymes that is, α-L-arabino-furanosidases, α-glucuronidases and acetyl esterases (Poutanen et al., 1987). Many of the xylanase producing microorganisms express multiple iso-forms that have been ascribed to a variety of reasons that is, heterogeneity and complexity of xylan structure (Banerjee et al., 1995). A large variety of microorganisms, including bacteria, yeast and filamentous fungi have been reported to produce xylanase (Subramaniyan and Prema, 2000). Filamentous fungi are industrially important producers of xylanase due to higher yield compared to yeast and bacteria (Haltrich et al., 1996). Xylanases are produced by either solid state or submerged fermentation. Enzyme productivity in solid state fermentation (SSF) is usually much higher than that of submerged fermentation (Rawashdeh et al., 2005). Therefore, solid state fermen-tation has gained interest from researchers in recent

---
*Corresponding author. E-mail: rajashri.kamble@gmail.com.
Tel: 02328-224012, 224013. Fax: 02328-223507.

years and has often been employed for the production of xylanases because of economic and engineering advantages (Aguilar et al., 2008).

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 (for example, manganese peroxidase, laccase) or by using hemicellulolytic enzymes (Pandey et al., 1999). Recently, xylanolytic enzymes are receiving increasing attention because of their potential applications in improving digestibility of animal feed, bioconversion of lingo-celluloses into feed-stocks, fuels and in pretreatment of pulps prior to bleaching in pulp and paper industry (Salles et al., 2005).

Main objectives of the study included isolation of potential xylanase-producing fungi from compost soil and to identify strains that secrete the maximum amount of xylanase during fermentation. The work also aimed to isolate fungal strains that could produce cellulase-free xylanase under solid state and submerged fermentation conditions. In the present investigation, fungal strains of various genera *Hamigera, Rhizopus, Aspergillus* and *Penicillium* were isolated and screened for xylanase activity.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used were of analytical grade. Bovine serum albumin, Sabouraud's dextrose agar, Czapek's agar medium and carboxy methyl cellulose were obtained from Hi-media, Mumbai, India. Cibacron brilliant red 3BA and birch wood xylan was purchased from Sigma chemicals Co., USA.

Isolation and screening of fungi

Two study sites Kolhapur (Western Maharashtra, 16°41'N 74.13°E) and Kagal (Western Maharashtra, 74°32'E 16°58'N) were randomly chosen, which lies on the Western Ghats of Maharashtra. Composting soil samples were collected after removing about 3 cm of soil from the surface. Each soil sample was crushed, mixed thoroughly and sieved through a 2 mm sieve (Rawashdeh et al.*,* 2005). The soil samples were serially diluted in sterile distilled water. Higher dilution of each sample was inoculated in sabouraud's dextrose medium containing 0.7% birchwood xylan. The plates were incubated at 28 ± 02°C for seven days. The fungal strains were transferred to fresh sabouraud dextrose agar plates containing 0.7% (w/v) birch wood xylan until pure cultures were obtained. The fungal isolates were sub-cultured to purity and were preserved on sabouraud dextrose agar slants under refrigeration conditions. The identification of fungal cultures was done according to Fisher and Cook (1998) and on the basis of 18S rDNA sequence.

Screening for xylanolytic activity on Czapek's agar medium

Eight fungal isolates were screened by xylan-agar diffusion method for their abilities to produce extracellular xylanase during their growth on enriched Czapek's agar medium containing xylan-red as the substrate (Nakamura et al., 1993). Xylan-red, a cross-linked xylan covalently coupled with cibacron brilliant red 3B-A, was prepared by the method reported (Ten et al., 2003). The composition of the medium was (gL^{-1}): xylan-red, 1.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 0.2 and agar 20.0. The inoculated plates were incubated for 7 days at 28 ± 2°C. Xylanolytic activity of each colony was detected by determining ratio of diameter of clearance zone (CZ) to the diameter of size of colony (CS).

Xylanase production under solid state fermentation

The selected strains were further tested for their abilities to produce extracellular xylanase under solid state fermentation. Wheat bran was used as the substrate. The strains were cultured in Erlenmeyer flasks (250 ml) containing 10 g of wheat bran moistened with 18 ml of the basal salt solution (BSS: substrate to moisture ratio 1:1.8). The composition of the basal salt solution was (gL^{-1}) NaCl, 30.00; KCl, 0.75; $MgSO_4$, 7.00; NH_4Cl, 0.5; K_2HPO_4, 2.5; KH_2PO_4 0.5; trace metal solution, 1.00 ml; distilled water, 1.0. pH of the medium was adjusted to 5.5. Composition of the trace metal was H_3BO_3, 2.85; $MnCl_2.7H_2O$, 1.80; $FeSO_4.7H_2O$, 2.49; Na–Tartarate, 1.77; $CuCl_2$, 0.03; $ZnCl_2$, 0.02; $COCl_2$, 0.04; $Na_2MoO_4.2H_2O$, 0.02; distilled water, 1.0 L. The media were then autoclaved at 121°C (15 lbs) for 20 min, cooled and inoculated with fungal spores. Seven days old fungal spores were harvested by aseptically adding sterile saline water containing 0.01% (v/v) Tween 80 so as to get the final concentration 1 × 10^6 spores ml^{-1}. 1 ml spore suspension was used as inoculum. After mixing, the flasks were incubated at 28 ± 2°C temperature for 7 to 14 days under static conditions. After incubation, at 12 h interval, samples were removed over a period of seven to 14 days. At each time interval 3 g of spent solid substrate was removed and suspended in 30 ml of 50 mM phosphate buffer (pH 8.0), vortexed thoroughly to extract the xylanase. The sample was centrifuged at 5000 x *g* for 10 min at 4°C (centrifugation will remove xylanase from substrate). Supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude xylanase preparation. Prior to centrifugation, the samples were withdrawn for determining viable number of cells by standard viable plate count technique.

Xylanase production under submerged fermentation

The composition of mineral salts medium was the same as that of the solid state fermentation with birch wood xylan as the carbon source. However, wheat bran was not added. The pH of the medium was adjusted to 5.0. 50 mL of the medium was transferred into a 250 ml Erlenmeyer flask, and after autoclaving was inoculated with 1 ml of spore suspension containing 1×10^6 spores mL^{-1}. The flasks were incubated at 28 ± 2°C on a rotary shaker (100 rpm) for seven to 14 days. After incubation, at 12 h interval, samples were removed over a period of 7 to 14 days and filtered through Whatman no.1 filter paper. The filtrate was centrifuged at 5000 x *g* for 10 min at 4°C. The clear supernatant was used as source of xylanase.

Enzyme assays

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) birch wood xylan (prepared in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of suitably diluted enzyme and the mixture was incubated at 50°C for 5 min (Bailey et al., 1992). The reaction was stopped by addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and the contents was boiled for 5 min (Miller, 1959). After cooling, the colour developed was read at 540 nm. The amount of reducing sugar

liberated was quantified using xylose as standard. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 mol of xylose equivalents per minute under the assay conditions.

Cellulase (CMCase) activity was determined by mixing 0.9 ml of 1% (w/v) CMC (prepared in 50 mM Na-citrate buffer pH 5.3) with 0.1 ml of suitably diluted enzyme and incubating at 50°C for 15 min (Ghose, 1987). The reaction was stopped by addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and the contents was boiled for 5 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 activity was defined as the amount of enzyme that liberates 1 mol of glucose equivalents per minute under the assay conditions.

Determination of soluble protein

The concentration of soluble protein was estimated using bovine serum albumin as the standard (Lowry, 1951).

Mycelial dry weight

Mycelial dry biomass was collected on a pre-weighed Whatmann filter paper no. 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

Data were expressed as mean ± standard deviation for all experiments and statistical significance was calculated according to student two-tailed 't test'. Values corresponding to $p < 0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

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 composting soil as they are rich sources of cellulose, hemicellulose (xylan 20 to 40%) and lignin. During the initial screening, a total of eight different fungal strains were isolated on sabouraud's dextrose agar medium. Identification of fungal strains was made on the basis of morphological, cultural and reproductive characteristics by using standard reference manuals of Fisher and Cook (1998) and also on the basis of 18S rDNA sequence. 18S rDNA sequences of fungal strains were deposited at the National Centre for Cell Sciences (NCCS), Ganesh Khind, Pune, India with assigned NCBI sequence accession numbers. Fungal strains were identified as *Hamigera insecticola* NRRL 35446 (EF634420.1); *Rhizopus* sp*.* ACCC 30795 (EF623861); *Aspergillus niger* 6 (HM347449); *Aspergillus flavus* UAF-138 (FJ969193); *Penicillium citrinum* CBMAI

853 (FJ790883); *Rhizopus microsporus* CB-10 (GU932682); *Rhizopus oryzae* NRRL 1526 (HM209240); *Penicillium* sp*.* J1 (HQ407379) as given in Table 1.

Among the eight isolates, 33% belong to *Rhizopus* species, forming a dominant group, followed by *Penicillium* species and *Aspergillus* species. Among the fungal strains identified, one belongs to *Hamigera* species. All the fungi were screened for extracellular xylanase activity on enriched Czapek's agar medium containing xylan-red as sole carbon source as shown in Figure 1.

For all the eight isolates, both qualitative (Cz/Cs) and quantitative (U ml^{-1}) 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 ratio of Cz/Cs ranged from 5.0 (*Aspergillus niger* 6) to 17.5 (*Hamigera insecticola* NRRL 35446 and *Aspergillus flavus* UAF-138). *Rhizopus* sp. ACCC 30795, *Penicillium citrinum* CBMAI 853 and *Penicillium sp.* J1 secreted substantial levels of xylanase enzyme which ranged from 12.5 to 10.0. The two *Rhizopus* species that is, *Rhizopus microsporus* CB-10 and *Rhizopus oryzae* NRRL 1526 showed moderate Cz/Cs ratio (6.6 and 8.3).

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). 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 = 0.223) 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 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.

The technique of solid-state fermentation involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water. These fermentation systems, which are closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites (Babu and Satyanarayana, 1995; Qadeer et al., 1980). SSF offers distinct advantages over submerged fermentation including economy of the space, simplicity of the media, no complex machinery, equipments and control systems, greater compactness of the fermentation vessel, greater product yields, reduced energy demand, easier scale up of processes and easier control of contamination due to the low moisture level in the system (Raimbault, 1998; Aguilar et al., 2008). The solid-state xylanase production was much better than submerged one. Hence xylanase production was compared in both the conditions.

In the present study, in solid state conditions, 50% of the selected strains could produce cellulase along with xylanase (Table 1). *Rhizopus oryzae* NRRL 1526 (923.0 U gds^{-1}) was the best xylanase producer, followed by *Rhizopus microsporus* CB-10 produced maximum amount of cellulase free xylanase (912.0 U gds^{-1}). *Rhizopus* sp. ACCC 30795 produced lower amount of xylanase (45.78 Ugds^{-1}). Among *Aspergilli, Aspergillus niger* 6 produced highest amount 822 U gds^{-1} xylanase and *Penicillium citrinum* UAF-138 produced 676.0 U gds^{-1} xylanase. *P. citrinum* CBMAI 853 and *Penicillium sp.* J1 produced 745.0 U gds^{-1} and 782.0 U gds^{-1} of xylanase respectively. *Hamigera insecticola* NRRL 35446 produced lower amount 65.0 U gds^{-1} of xylanase.

Xylanase in combination with cellulase 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 (Pandey et al., 1999). Therefore, to select potential cellulase-free xylanase producing fungi, both enzyme (xylanase and cellulase) activities were measured.

Concomitant cellulase production with xylanase was monitored during solid state fermentation and four fungal strains could produce cellulase along with xylanase. This might be because of the presence of cellulose in wheat bran, the substrate for solid-state. Previously, it was reported that both xylanase and cellulase was produced when cellulose and hemicellulose were used together as carbon source (Kulkarni et al., 1999). Haltrich et al. (1996) reported that xylan-degrading organisms are often cellulolytic and secrete complex mixtures of xylanases and cellulases concurrently.

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 = 0.051) 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 *Aspergillus niger* 6 (2.90 mg ml^{-1}), while the lowest was noticed for *Hamigera insecticola* NRRL 35446 (0.70 mg mL^{-1}). 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 eight fungi was calculated and the range was between 67.0 mg gds^{-1} (*Rhizopus* sp. ACCC 30795) to 99.0 mg gds^{-1} (*Rhizopus oryzae* NRRL 1526).

Xylanase production in solid-state was much higher than that in submerged fermentation. Kheng and Ibrahim (2005) observed xylanase production increased by 157% in SSF system when palm kernel cake was used as the substrate for *Aspergillus niger* USM A1 I, while Malarvizhi et al. (2003) observed 30-fold enhancement of xylanase production in solid state fermentation than liquid culture when wheat bran was used as the substrate for a culture of *Ganoderma lucidum*.

Xylanase is an inducible enzyme and xylan present in lignocellulosic waste acted as better inducer for xylanase production. Haltrich et al. (1996) reported that addition of small amounts of purified xylan to complex lignocellulosic substrates like wheat bran resulted in considerable enhancement of xylanase production. Wheat bran proved to be a suitable substrate along with 0.1% (w/v) birch wood xylan for the production of xylanase during solid state. Several workers reported the suitability of wheat bran for xylanase production by solid-state (Gawande and Kamat, 1999; Malarvizhi et al., 2003).

Among eight fungal strains, six could produce cellulase-free xylanase under submerged conditions (Table 2). Among all strains, *Rhizopus oryzae* NRRL 1526 (205.0 U ml^{-1}) and *Rhizopus microsporus* CB-10 (126.0 U ml^{-1}) were the most prominent cellulase-free xylanase producers. Other showed moderate xylanase production as *Hamigera insecticola* NRRL 35446 (66.0 U mL^{-1}), *Rhizopus* sp. ACCC 30795 (75.10 U ml^{-1}), *Aspergillus niger* 6 (76.0 U ml^{-1}), *Aspergillus flavus* UAF-138 (48.0 U ml^{-1}), *Penicillium citrinum* CBMAI 853 (63.0 U ml^{-1}) and *Penicillium sp.* J1 (45.0 U ml^{-1}). The above experimental values were much closer to the results

Table 1. The qualitative and quantitative assay of xylanases by isolated eight fungal strains in solid state fermentation.

S/N	Isolate identity	Name of the fungal species	Qualitative assay		pH	Quantitative assay			Mycelial dry weight (mg ml^{-1})
			Hydrolyzed zone (Cz/Cs)			Xylanase (U gds^{-1})	Cellulase (U gds^{-1})	Protein (mg gds^{-1})	
1	KOL2	*Hamigera insecticola* NRRL 35446	17.5		5.0	65.0 ± 10.0	-	80.0 ± 15.50	0.70
2	KOL5	*Rhizopus* sp. ACCC 30795	11.33		5.5	45.78 ± 5.4	-	67.0 ± 7.60	0.90
3	K 1	*Aspergillus niger* 6	5.0		6.0	822.0 ± 24.5	33.0 ± 0.33	95.0 ± 8.33	2.90
4	K 2	*Aspergillus flavus* UAF-138	17.5		5.0	676.0 ± 20.0	26.0 ±10.41	86.0 ± 13.4	1.10
5	K 3	*Penicillium citrinum* CBMAI 853	12.5		5.0	745.0 ± 35.6	20.0 ± 3.55	76.0 ± 2.1	1.0
6	K 5	*Rhizopus microsporus* CB-10	6.6		5.0	912.0 ± 45.50	-	90.0 ± 21.1	0.89
7	K 7	*Rhizopus oryzae* NRRL 1526	8.3		5.0	923.0 ± 50.0	-	99.0 ± 12.20	1.50
8	K 8	*Penicillium* sp. J1	10.0		5.0	782.0 ± 18.8	20.0 + 0.98	93.0 ± 10.90	1.21

U gds^{-1}, Unit per gram dry substance

Table 2. The quantitative assay of xylanases and cellulases by isolated eight fungal strains in submerged fermentation.

S/N	Isolate identity	Name of the strain	pH	Quantitative assay		
				Xylanase (U mL^{-1})	Cellulase (U mL^{-1})	Protein (µg mL^{-1})
1	KOL2	*Hamigera insecticola* NRRL 35446	5.0	66.0 ± 13.33	-	65.0 ± 12.40
2	KOL5	*Rhizopus* sp. ACCC 30795	5.5	75.10 ± 10.0	-	45.0 ± 5.6
3	K 1	*Aspergillus niger* 6	6.0	76.0 ± 16.50	13.0 ± 1.60	67.0 ± 7.90
4	K 2	*Aspergillus flavus* UAF-138	8.0	48.0 + 12.45	12.0 ± 3.5	78.0 ± 18.8
5	K 3	*Penicillium citrinum* CBMAI 853	5.0	63.0 ± 5.6	-	79.0 ± 8.0
6	K 5	*Rhizopus microsporus* CB-10	5.0	126.0 ± 42.10	-	85.0 ±12.0
7	K 7	*Rhizopus oryzae* NRRL 1526	5.0	205.0 ± 15.7	-	64.0 ± 6.7
8	K 8	*Penicillium* sp. J1	5.0	45.0 ± 3.2	-	68.0 ± 10.90

obtained by Costa- Ferreira et al. (1994) in shake flask using *Aspergillus niger* isolates. 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 eight fungi was calculated and the range was between 45.0 µg ml^{-1} (*Rhizopus* sp. ACCC30795) to 85.0 µg ml^{-1} (*Rhizopus microsporus* CB-10).

In the present study, cellulase production was almost absent in submerged fermentation. In submerged condition pure xylan was the only substrate with absence of any kind of cellulose source. 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). Haltrich et al. (1996) reported that purified xylan was good enhancer of xylanase production. Similar type of studies on xylanase production under submerged fermentation had been performed by Li et al. (2007) and Wang et al.
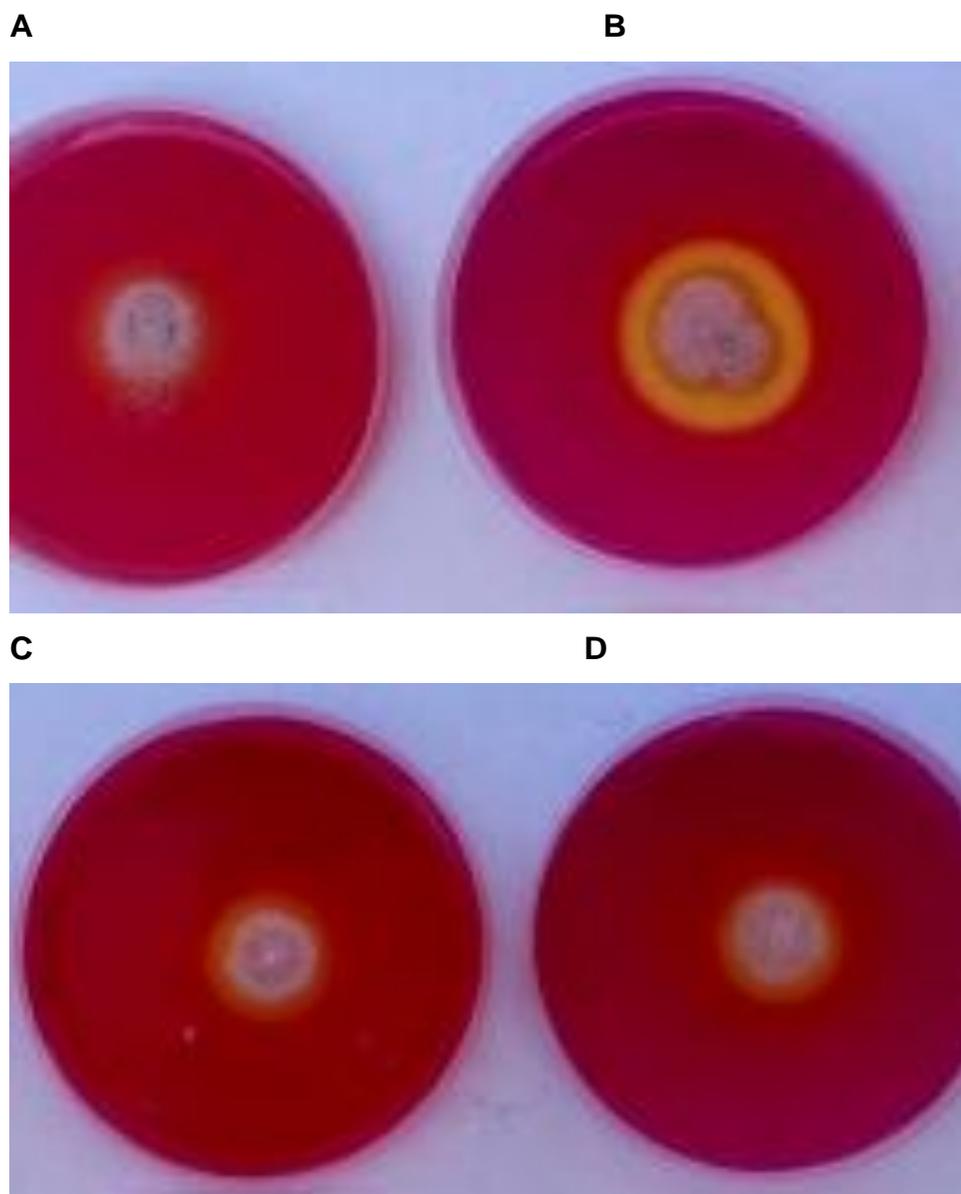
Figure 1. Colonial growth of isolated fungal strains on xylan-red enriched Czapek's agar medium. **A.** *Hamigera insecticola* **B.** *Rhizopus* sp*.* **C.** *Aspergillus niger* **D.** *Penicillium* sp.

(2010).

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 fermen-tation 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.

ACKNOWLEDGEMENTS

REFERENCES

Aguilar CN, Gerardo GS, Plilia ARB, Raul RH, Jose LMH, Juan CCE (2008). Perspectives of solid state fermentation for production of food enzymes. Am. J. Biochem. Biotech. 4(4):354-366.

Anthony T, Chandra Raj K, Rajendran A, Gunasekaran P (2003). High molecular weight cellulase-free xylanase from alkali-tolerant Aspergillus fumigatus AR1. Enzyme Microb. Technol. 32(6):647-654.

Babu KR, Satyanarayana T (1995). α-Amylase production by thermophilic Bacillus coagulans in solid state fermentation. Process Biochem. 30:305-309.

Bailey M J, Biely P, Poutanen K (1992). Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23:257-270.

Banerjee S, Archana A, Satyanarayana T (1995). Xylanolytic activity and xylose utilization by thermophilic molds. Folia Microbiol. 40(3):279-282.

Biely P (1985). Microbial xylanolytic systems. Trends Biotechnol. 3(11):286-290.

Biswas SR, Jana SC, Mishra AK, Nanda G (1990). Production, purification, and characterization of xylanase from a hyperxylanolytic mutant of Aspergillus ochraceus. Biotechnol. Bioeng. 35(3):244-251.

Costa-Ferreira M, Diass A, Maximo C, Morgado MJ, Sena-Martins G, Duarte JC (1994). Xylanolytic enzyme production by an Aspergillus niger isolate. Appl. Biochem. Biotechnol. 44:231-242.

Fisher F, Cook NB (1998). Some opportunistic fungi. In: Fundamentals of Diagnostic Mycology WB Saunders company (Publ). pp. 35-102.

Flannigan B, Gilmour EMJ (1980). A simple plate test for xylanolytic activity in wood-rotting Basidiomycetes. Mycologia 72(6):1219-1221.

Gawande PV, Kamat MY (1999). Production of Aspergillus xylanase by lignocellulosic waste fermentation and its application. J. Appl. Microbiol. 87:511-519.

Gilbert M, Breuil C, Saddler JN (1992). Characterization of the enzymes present in the cellulase system of Thielavia terrestris 255B. Biores. Technol. 39(2):147-153.

Ghose TK (1987). Measurement of cellulase activities. Pure Appl. Chem. 59:257-268.

Ghosh M, Nanda G (1994). Physiological studies on xylose induction and glucose repression of xylanolytic enzymes in Aspergillus sydowii MG49. FEMS Microbiol. Lett. 117(2):151-156.

Haas H, Herfurth E, Stöffler G, Redl B (1992). Purification, characterization and partial amino acid sequences of a xylanase produced by Penicillium chrysogenum. Biochim. Biophys. Acta (BBA)-General Subjects 1117(3):279-286.

Haltrich D, Nidetzky B, Kulbe KD, Steiner W, Zupancic S (1996). Production of fungal xylanases. Biores. Technol. 58:137-161.

Hrmova M, Petrakova E, Biely P (1991). Induction of cellulase- and xylan-degrading enzyme systems in Aspergillus terreus by homoand heterodisaccharides composed of glucose and xylose. J. Gen. Microbiol. 137(3):541-547.

Kheng PP, Ibrahim CO (2005). Xylanase production by a local fungal isolate, Aspergillus niger USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin J. Sci. Technol. 27(2):325-336.

Kulkarni N, Shendye A, Rao M (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 23:411-456.

Li Y, Zhiqiang L, Fengjie C, Yingying X, Hui Z (2007). Production of xylanase from a newly isolated Penicillium sp. ZH-30. World J. Microbiol. Biotechnol. 23:837-843.

Lowry OH (1951). Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Malarvizhi K, Murugesan K, Kalaichelvan PT (2003). Xylanase production by Ganoderma lucidum on liquid and solid state fermentation. Indian J. Exp. Biol. 41:620-626.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(3):426-428.

Nair SG, Sindhu R, Shashidhar S (2008). Fungal xylanase production under solid state and submerged fermentation condition. Afr. J. Microbiol. Res. 2:82-86.

Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993). Purification and some properties of an alkaline xylanase from alkaliphilic Bacillus sp. strain 41 M-1. App. Environ. Microbiol. 59(7):2311-2316.

Palma MB, Ferreira Milagres AM, Prata AMR, de Mancilha IM (1996). Influence of aeration and agitation rate on the xylanase activity from Penicillium janthinellum. Process Biochem. 31(2):141-145.

Pandey A, Selvakumar P, Soccol CR, Nigram P (1999). Solid state fermentation for the production of industrial enzymes. Curr. Sci. 77:149-162.

Poutanen K, Ratto M, Puls J, Viikari L (1987). Evaluation of different microbial xylanolytic systems. J. Biotechnol. 6:49-60.

Qadeer MA, Anjum JI, Akhtar R (1980). Biosynthesis of enzymes by solid substrate fermentation. Part II: Production of alpha-amylase by Bacillus subtilis. Pak. J. Sci. Ind. Res. 23:25-29.

Raimbault M (1988). General and microbiological aspects of solid substrate fermentation. Electron. J. Biotechnol. 1(3):175-188.

Rawashdeh R, Saadoun I, Mahasneh A (2005). Effect of cultural conditions on xylanase production by Streptomyces sp. (strain LB 24 D) and its potential to utilize tomato pomace. Afr. J. Biotechnol. 4(3):251-255.

Salles BC, Medeiros RG, Bao SN, Silva Jr. FG, Filho EXF (2005). Effect of cellulase-free xylanases from Acrophialophora nainiana and Humicola grisea var. thermoidea on eucalyptus kraft pulp. Process Biochem. 40:343-349.

Subramaniyan S, Prema P (2000). Cellulase free xylanases from Bacillus and other microorganisms. FEMS Microbiol. Lett. 183:1-7.

Teather RM, Wood PJ (1982). Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43(4):777-780.

Ten LN, Im WT, Kim MK, Kanga MS, Lee ST (2003). Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. J. Microbiol. Meth. 56:375-382.

Tlecuitl-Beristain S, Sanchez C, Loera O, Robson GD, Diaz-Godínez G (2008). Laccases of Pleurotus ostreatus observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform. Mycol. Res. 112(9):1080-1084.

Tseng YH, Fang TJ, Tseng SM (2000). Isolation and characterization of a novel phytase from Penicillium simplicissimum. Folia Microbiol (Praha). 45(2):121-127.

Wang CY, Chan H, Lin HT Shyu YT (2010). Production, purification and characterisation of a novel halostable xylanase from Bacillus sp. NTU-06. Ann. Appl. Biol. 156:187-197.