Singh et al., 2:1
http://dx.doi.org/10.4172/scientificreports.602

Open Access Scientific Reports

Research Article Open Access

Screening of Xylanases from Indigenously Isolated White Rot Fungal Strains for Possible Application in Pulp Biobleaching

Shalini Singh[1]*, Dharm Dutt[2] and CH Tyagi[2]

Lovely Professional University, Punjab, India
[2]Department of Paper Technology, IIT Roorkee, Saharapur Campus, India

Abstract

The current study aimed at screening indigenously isolated white rot fungi strains for xylanase production, for potential application in pulp biobleaching. White rot fungi have effective hemicellulase systems. Still, only a few studies have been made on their hemicellulases. 14 white rot basidiomycetous strains were isolated, and were found to exhibit variable xylanase activity, as indicated by the zone of clearance on wheat bran agar medium. Out of the 14 xylanase positive isolates, seven showing zone of clearance greater than equal to 2 cms, were subjected to fermentation under SSF and LSF, so as to determine their actual enzyme activities. Isolate C and E showed the highest xylanase activity (697 IU/mL, (131.25 IU/mL, respectively), followed by D (94 IU/mL)>B (80.41 IU/mL)>F (54.89 IU/mL)>G (17.90 IU/mL) >A (4.51 IU/mL), after incubation period of 7 days. All the 7 test isolates were found to produce laccases and minimal amount of cellulases. Solid State Fermentation (SSF) reported much better xylanase production, as compared to Liquid State Fermentation (LSF), indicating that SSF was the preferred mode of cultivation for the test isolates. Wheat bran agar medium was found to be the most suitable for the growth of the two best xylanase producers. The results indicated that the isolates showing good xylanase activity, with poor cellulase activity can very well be used in pulp biobleaching. The production of laccases by the test isolates also is preferable, as it further enhances the applicability of the isolates in pulp biobleaching.

Keywords: Xylanase; White rot fungi; SSF, LSF; Biobleaching

Introduction

There has been an unprecedented expansion in our knowledge of the use of microorganisms, their metabolic products, and enzymes in a broad area of basic research, and their potential industrial applications. Xylanolytic enzymes have attracted a great deal of attention, and are applied in manufacturing of bread, food and drinks, improvement of nutritional properties of agricultural silage and grain feed, textile industry to process plant fibres, pharmaceutical and chemical applications, and cellulose pulp and paper [1,2]. Recently, the interest in xylanases has focused on bleaching processes [3-5], wherein, enzymes began to be used during the last two decades, ever since peroxidases were applied to the degradation of lignin [6,7]. Enzyme application improves pulp fibrillation and water retention, reduces beating time in virgin pulps, restores bonding and increased freeness in recycled fibres, and selectively removes xylans from dissolving pulps. The application of xylanases in prebleaching of pulps is gaining importance as alternatives to toxic chlorine-containing chemicals [8-10], where xylanases offer an attractive and commercially viable option to eliminate chlorine in bleaching, and reduce chlorinated organic compounds in bleach plant effluents, reduce the kappa number (residual lignin content in the pulp), and increase the brightness of the pulp [11,12]. Xylanases are being tested as bleaching agents for a variety of wood and non-wood raw materials [13]. The presence of cellulases in such preparations has been considered detrimental to yield and strength properties in pulp treatments, as they can cause a rapid depolymerization of cellulose [14]. However significant differences in the action of individual cellulases in enzyme prebleaching have been observed.

The most potent producers of xylanases are the fungi [15], especially, wood-rot fungi may serve as good producers of xylanases, as well as other extracellular polysaccharides-degrading enzymes, since they excrete the enzymes into the medium, and their enzyme levels are much higher than those of yeast and bacteria [16]. Both the so-called brown and soft rot fungi decompose principally the polysaccharides. A third group, the wood-rotting basidiomycetes causing white-rot decay is able to degrade all the components of the wood cell wall, including the highly recalcitrant polymer, lignin [17]. Only a few studies have been made on the hemicellulases of white-rot fungi, but it is clear from their ability to deplete all the structural components of wood and from studies demonstrating growth on hemicelluloses substrates, that white-rot fungi have effective hemicellulase systems [18].

Since biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is the search for powerful xylanase producers, and the utilization of lignocellulosic wastes/by-products which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis, ensuring efficient production of lignocellulolytic enzymes, thereby exterminating the need of expensive pure xylans as substrate [19].

The present work aims at screening of indigenously isolated potent xylanase producing wood rotting basidiomycetes, for selecting efficient xylanase producers for possible application in pulp biobleaching, using cheap lignocellulosic substrates.

Materials and Methods

Birchwood xylan and syringaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA), 3,5-Dinitrosalicylic acid (DNS), was procured from Loba Chemie, India. All other reagents were of analytical grade, made by known manufacturers. Wheat bran, rice

***Corresponding author:** Shalini Singh, Lovely Professional University, Punjab-144402, India, Tel: +919501445358; E-mail: shalinisingh.iit@gmail.com

Received November 21, 2012; **Published** january 06, 2013

Citation: Singh S, Dutt D, Tyagi CH (2013) Screening of Xylanases from Indigenously Isolated White Rot Fungal Strains for Possible Application in Pulp Biobleaching. 2: 602 doi:10.4172/scientificreports.602

bran, sugarcane was procured from local market of Saharanpur, Uttar Pradesh, India, washed two to three times with warm water, and then immediately dried in sunlight. The dried substrate was grinded in a laboratory grinder, to obtain a fine powder. The fine powder was passed through 100 µm mesh size screen, and the fractions so obtained were stored in polyethylene bags for further use.

Isolation and microscopic examination of xylanase producing basidiomycetes

Basidiomycetous strains were isolated from decaying wood, from various locations in Saharanpur region of Uttar Pradesh, India, by enrichment technique, where dead and decaying wood samples were collected and kept in glass Petri plates, containing moist wheat bran. The plates were incubated at 37°C for 2-10 days. The moisture level was carefully controlled with sterile water, so as to provide a solid substrate for fungal growth, with no free water available. The plates were observed for the appearance of fungal growth. The fungal strains were purified by aseptically transferring mycelia from the inner part of the fruiting bodies onto wheat bran agar medium (2% w/w wheat bran, 2% agar-agar and 185 µg/mL of chloramphenicol) [20]. The plates were incubated at 37°C for 72 h. The purified cultures were routinely cultured on Potato Dextrose Agar (PDA) slants, with incubation at 37°C for 72 h, and subsequently stored at 4°C. The cultures were maintained as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at -20°C, for long term preservation.

The fungal isolates so obtained, were subjected to microscopic examination, wherein the fungal mycelium was picked, kept on a microscopic slide, and a drop of lacto-phenol cotton blue stain was added to it. A cover slip was gently placed over the specimen. The fruiting bodies were picked, kept on a microscopic slide in a similar fashion, and pressed with gentle pressure of the thumb, after placing a cover slip over the specimen. The microscopic features of the fungal isolates were also studied using microscopic slides containing a smear of PDA. PDA was first prepared and autoclaved. A smear of this medium was prepared on autoclaved microscopic slides. These slides were then point inoculated with the test fungi and incubated at 37°C, until desired growth appeared. All the slides were viewed under light microscope, and microphotographs were taken under different magnifications.

Primary screening for xylanase, cellulase and laccase production

The fungal isolates were tested for xylanase production by growing them on xylan-agar medium, containing 1% w/v birch wood xylan and 2% agar agar [21] at 37°C. One 5 mm disc from 4-day-old fungal cultures was inoculated in this medium and the plates were incubated at 37°C. After 6 days of incubation period, the plates were stained with Congo red solution (0.5% w/v Congo red and 5% v/v ethanol in distilled water) for 15 min, and then destained with 1M NaCl. The plates were then observed for appearance of clear zones around the fungal cultures against the red background [22].

The cellulase (CMCase) assay [22] for fungal strains was done by inoculating one 5 mm fungal discs in minimal medium (0.25% CMC, and 1.5% agar). After incubation for 6 days at 37°C, the Petri plates were flooded with 0.1% Congo Red for 15 minutes, and subsequently destained with 1M NaCl for 30 minutes. Zone of clearance were observed around the fungal growth.

The test fungi were also tested for their abilities to produce laccase by laccase plate assay, in which they were grown on guaiacol-agar medium (0.02% w/v guaiacol, 1% w/v yeast extract, and 2% w/v agar agar). 5 mm discs from 4 day old cultures of the test fungi were inoculated in this medium, and the plates were then incubated at 37°C for 10 days to get sufficient growth of the cultures on the medium.

Enzyme production under different modes of cultivation (solid state fermentation, SSF, and Liquid State Fermentation, LSF)

5 g wheat bran was added in Erlenmeyer flask (Borosil) of 250 mL capacity, to which 15 mL of nutrient salt solution [23,24], maintained at desirable pH was added [25]. The flasks were autoclaved, cooled, and inoculated aseptically with 2 discs (5 mm diameter each), of 3-day-old culture of each fungal isolate. The inoculated flasks were incubated at desired temperature and harvested after desired incubation period.

As in SSF, enzyme production under LSF was also carried out in 250 mL Erlenmeyer flasks (Borosil). 0.8 g powder of wheat bran was added in each flask, to which 40 mL of NSS, maintained at desirable pH [23,24], was added. The above-prepared flasks were plugged with cotton and sterilized at 121°C, 15 Psi for 15 min. When cooled at room temperature, the pH of flasks were checked and readjusted aseptically. Two discs of 5 mm diameter from 4-day-old culture of test isolate were aseptically inoculated in each of the flask. These flasks were incubated in incubator shaker (Sanyo, Orbi-safe, UK), at 100 rpm at desired temperature and incubation period.

Harvesting and storage of enzyme

After desired growth of the test fungi under SSF, the enzyme was harvested. 15 mL of distilled water was added to each flask. The contents of the flask were crushed with the help of a glass rod, and were then shaken on orbital shaker at 100 rpm, for 10 min at room temperature. The contents were filtered through the four layers of cheese cloth. The filtrate was centrifuged at 5000×g (Sigma laboratory centrifuge model 3-18K) [26], for 10 min at 4°C. The supernatant liquid was treated as crude enzyme and stored at -20°C in small volumes, until use.

The enzymes produced under LSF were harvested in the similar manner as in SSF, with the difference that no distilled water was added in the flasks, and the contents of flasks were directly filtered through cheese cloth.

Estimation of enzyme activities

The xylanase activity was determined by measuring the release of reducing sugars, using birch wood xylan (Sigma Chemicals Co.) as substrate by 3, 5-Dinitrosalicylic acid (DNS) method [27], at 55°C for 15 min, with constant shaking at 100 rpm. Optical density was measured at 540 nm in a double beam UV–vis spectrophotometer (Systronics UV–vis double beam spectrophotometer model-2201). One unit of activity was defined as the amount of enzyme needed to release one mmol of xylose equivalents released per min at 55°C.

Likewise, CMCase activity was determined by incubating 2 mL of enzyme preparation with 2 mL of 2% (w/v) Carboxymethyl Cellulose (CMC) (Qualigens Fine Chemicals, Mumbai) [28], prepared in 0.05 M citrate buffer (pH-4.8) at 50°C for 30 min. The reducing sugars were measured by DNS method [27] at 575 nm, and expressed as glucose equivalent. The enzyme activity was expressed as mmoles of D-glucose equivalents released per min at 50°C and pH-4.8 (IU).

The laccase activity in the enzyme samples was determined by continuous spectrophotometric rate determination method given by Ride [29]. The enzyme assay is based on the oxidation of syringaldazine by laccase, to form oxidized syringaldazine. In this method, sets of test

samples containing 2.2 mL of reagent 'A' (100 mM potassium phosphate buffer, pH 6.5 at 30°C) and 0.5 mL reagent 'C' (enzyme solution prepared at suitable concentration in cold deionized water), and blank solutions containing 0.5 mL deionized water and 2.2 mL reagent 'A', were pipetted into suitable cuvettes. These cuvettes were equilibrated to 30°C, and wavelength at 530 nm (A_{530} nm) was monitored until constant, using a thermostatted spectrophotometer. After equilibration, 0.3 mL of reagent 'B' (0.216 mM syringaldazine solution) was added to each test sample and the blank solution. The contents in each cuvette were immediately mixed by inversion, and the increase in absorbance at 530 nm (A_{530} nm) was recorded for about 10 min. The change in A_{530} nm per min was obtained by using the maximum linear rate, for both the test and blank. One unit is defined as unit that will produce A_{530} nm of 0.001 per min at pH-6.5 at 30°C in a 3 mL reaction volume, using syringaldazine as substrate, and the enzyme activity was expressed as unit per mL of the sample.

Effect of medium composition on the growth of basidiomycetous isolates

The effect of different media composition on the growth of test fungi was studied by comparing their growth on complex nutrient media, as well defined nutrient media. The complex nutrient media used included Wheat Bran Agar (WBA) medium, containing 2 wheat bran and 2% agar agar; Rice Bran Agar (RBA) medium, containing 2% rice bran and 2% agar agar; Sugarcane Bagasse Agar (SBA) medium, containing 2% sugarcane bagasse and 2% agar agar. Defined nutrient media used included Xylan agar media, Potato Dextrose Agar (PDA), containing potato infusion 20%, dextrose 2% and 1.5% agar agar, Saboraud's Dextrose Agar (SDA), containing 1% peptone, 4% dextrose and 1.5% agar, Malt Extract Agar (MEA), containing, 1.5% malt extract, 0.01% Dipotassium hydrogen phosphate, 0.01% ammonium chloride, citric acid (N/1) 1.5 mL, 2% agar agar, and Czapek Dox Agar (CDA) media, containing 0.2% sodium nitrate, 0.01% dipotassium hydrogen phosphate, 0.005% magnesium sulfate, 0.005% potassium chloride, 0.0001% ferrous sulfate, 3% sucrose, 1.5% agar agar. 5 mm discs from 4 day old cultures of the test fungi were inoculated in the centre of these nutrient medium plates. The plates were incubated at 37°C, and growth was measured after 5 days.

Results and Discussion

Isolation and microscopic examination of xylanase producing basidiomycetes

Fourteen fungal strains were isolated from decaying wood samples by enrichment culture technique, where selective culture media and incubation conditions were used to isolate microorganisms directly from nature. In the present investigation, moist wheat bran, a promising carbon source for the production of lignocellulases [30], was used in the medium as sole carbon source. The isolates exhibited white thread like mycelial network on the decaying wood and aerial fruit bodies, after successive degradation, indicating the growth of basidiomycetous strains. These were isolated and purified on wheat bran agar medium. The purified isolates produced fruiting bodies under laboratory conditions. Brown coloured basidiospores were seen when mature fruiting bodies were observed under light microscope. This confirmed the isolates as white rot basidiomycetes.

Microscopic examination of the fungal isolates in a lactophenol-cotton blue mount revealed the presence of basidium, basidiospores, pileocystidea, hymenial layers, and chlamydospores (mitotic submerged spores). Clamp connections were also observed, when isolates were directly inoculated on a smear of PDA medium on microscopic slides. This is a characteristic feature of class *Hymenomycetes* of Basidiomycotina [31].

Primary screening for xylanase, cellulase and laccase production

All the 14 isolated white rot fungal strains (Table 1) were found to be xylanase positive, showing variable activities, as indicated by the areas of clear zones around the fungal growth, which in turn, showed solubilization of xylan due to the hydrolytic action of endoxylanases. The strains also showed variable Cellulase (CMCase) activity, as shown by the zone of clearance around fungal growth on Carboxymethyl Cellulose (CMC) agar medium. The isolates that showed good xylanase activity, as indicated by a zone of clearance of 2 cm or more, and poor/no cellulase activity, were selected for further studies. The idea was to select those fungal strains that showed promising xylanase activity and poor/no cellulase activity, so that the concerned enzyme preparation does not negatively affect pulp fibre integrity during biobleaching. Hence, a total of 7 isolates (Isolate No. 2, 3, 4, 5, 8, 10 and 11), were selected for further studies. Though isolate 9 showed good xylanase activity, yet it was not selected for further studies, as it also showed good cellulase activity. The fungal isolates were also found to be laccase positive. Though laccase activity was not considered as a prime criterion for the selection of isolates for further studies, yet the isolates were also tested for laccase production, as laccases can contribute towards further enhancing the biobleaching capabilities of the fungal strains.

Enzyme production under different modes of cultivation (SSF and LSF)

The fungal isolates were further subjected to Solid State Fermentation (SSF) conditions for a fixed incubation period, to determine the xylanase, cellulase, laccase activities, and supernatant protein concentrations of the crude enzyme preparations obtained from them (Table 2). The aim was to select isolates exhibiting the highest xylanase activities among the isolates, with minimal or no cellulase activities. This is desirable as presence of high amount of cellulases in the enzyme preparation might negatively influence the pulp yield, thereby, limiting the prospects of its use in pulp biobleaching for pulp and paper industry. The effect of mode of cultivation on enzyme production by

Sl. No.	Isolates	Xylanase assay	Cellulase assay	Laccase assay
1.	1	++	+++	+
2.	2	+++	+	+
3.	3	++++	+	++
4.	4	+++	++	+
5.	5	++++	+	++
6.	6	++	++	+
7.	7	+	-	+
8.	8	+++	++	+
9.	9	+++	++++	+
10.	10	+++	++	+
11.	11	+++	++	++
12.	12	+	++	++
13.	13	++	++	+
14.	14	++	+++	++

+ Very poor activity (<0.5 cm)
++ Poor activity (<0.5-2.0 cm)
+++ Good activity (2.0 cm)
++++ Very good activity (>2.0 cm)

Table 1: Plate assay for xylanase, cellulase and laccase production.

Incubation period	Fungal Isolates	Enzyme profile under different modes of cultivation					
		SSF			LSF		
		Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Laccase activity (U/mL)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Laccase activity (U/mL)
5	A	0.80 ± 0.04	0.08 ± 0.03	0.131 ± 0.05	0.666 ± 0.16	0.55 ± 0.16	0.100 ± 0.02
	B	58.32 ± 0.21	0.970 ± 0.26	0.028 ± 0.03	31.23 ± 3.11	0.83 ± 0.21	0.021 ± 0.03
	C	670.38 ± 10.17	0.601 ± 0.23	0.180 ± 0.04	40.60 ± 3.32	0.52 ± 0.09	0.100 ± 0.34
	D	55.60 ± 1.32	0.621 ± 0.03	0.478 ± 0.21	22.11 ± 3.00	0.40 ± 0.13	0.127 ± 0.05
	E	119.49 ± 5.33	0.580 ± 0.16	0.249 ± 0.03	28.98 ± 2.05	0.30 ± 0.05	0.176 ± 0.10
	F	50.02 ± 2.10	5.450 ± 1.01	0.021 ± 1.32	8.70 ± 1.01	4.21 ± 0.13	0.020 ± 0.01
	G	8.34 ± 1.12	2.542 ± 0.78	0.111 ± 0.07	2.92 ± 0.09	1.78 ± 0.21	0.980 ± 0.13
6	A	1.15 ± 0.14	0.141 ± 0.10	0.210 ± 0.10	0.750 ± 0.05	0.112 ± 0.06	0.198 ± 0.05
	B	80.41 ± 1.19	0.700 ± 0.26	0.234 ± 0.04	55.20 ± 1.32	0.521 ± 0.20	0.212 ± 0.03
	C	658.12 ± 11.18	0.940 ± 0.18	0.431 ± 0.13	72.10 ± 2.11	0.750 ± 0.12	0.187 ± 0.04
	D	91.48 ± 2.87	0.925 ± 0.13	0.700 ± 0.21	28.90 ± 1.59	0.800 ± 0.13	0.245 ± 0.10
	E	131.25 ± 3.15	0.712 ± 0.32	0.581 ± 0.20	49.25 ± 4.65	0.380 ± 0.15	0.301 ± 0.13
	F	54.89 ± 4.01	1.324 ± 0.21	0.100 ± 0.03	12.90 ± 0.21	0.890 ± 0.23	0.102 ± 0.05
	G	9.10 ± 0.42	1.101 ± 0.23	0.123 ± 0.05	3.21 ± 0.13	0.807 ± 0.26	0.100 ± 0.02
7	A	4.51 ± 1.01	0.298 ± 1.02	0.423 ± 0.05	3.00 ± 0.98	0.192 ± 0.07	0.410 ± 0.13
	B	70.43 ± 1.54	0.610 ± 0.03	0.332 ± 0.11	31.22 ± 5.03	0.459 ± 0.12	0.290 ± 0.10
	C	697.00 ± 0.21	0.802 ± 0.26	0.640 ± 0.10	97.90 ± 8.97	0.117 ± 0.02	0.590 ± 0.12
	D	94.00 ± 5.21	1.450 ± 0.23	0.932 ± 0.20	43.10 ± 4.33	0.421 ± 0.25	0.865 ± 0.33
	E	159.00 ± 5.20	0.500 ± 0.12	0.742 ± 0.12	64.87 ± 7.60	0.173 ± 0.04	0.675 ± 0.10
	F	30.00 ± 1.12	2.250 ± 1.32	0.321 ± 0.13	20.03 ± 1.56	0.979 ± 0.32	0.210 ± 0.05
	G	17.90 ± 2.03	0.532 ± 0.08	0.543 ± 0.22	7.70 ± 1.02	0.200 ± 0.03	0.444 ± 1.10
8	A	2.30 ± 0.13	0.100 ± 0.02	0.550 ± 0.41	1.87 ± 0.23	0.069 ± 0.03	0.223 ± 1.04
	B	53.00 ± 1.56	0.252 ± 0.09	0.100 ± 0.06	20.45 ± 2.45	0.201 ± 0.07	0.121 ± 0.06
	C	417.77 ± 7.14	0.410 ± 0.13	0.732 ± 0.22	69.92 ± 0.21	0.121 ± 0.03	0.700 ± 0.18
	D	71.00 ± 1.56	0.800 ± 0.20	0.989 ± 0.20	19.09 ± 2.12	0.321 ± 0.10	0.603 ± 0.25
	E	123.98 ± 4.10	0.456 ± 0.10	0.800 ± 0.21	31.00 ± 1.15	0.125 ± 0.03	0.752 ± 1.32
	F	28.01 ± 2.10	1.301 ± 0.21	0.330 ± 0.13	13.98 ± 1.56	1.002 ± 0.21	0.301 ± 0.13
	G	10.25 ± 0.98	0.200 ± 0.07	0.459 ± 0.05	4.09 ± 1.03	0.05 ± 0.02	0.367 ± 0.05
Fermentation conditions: Substrate: Nutrient salt solution=1:3 pH =6.0 Temperature, ºC=37					**Assay conditions:** Temperature, ºC=55 pH= 6.4 Incubation time, min=15		

± Standard deviation from the mean

Table 2: Enzyme production under different modes of cultivation (SSF and LSF).

the fungal isolates was also studied. When enzyme production by the test isolates was compared under different modes of cultivation, it was found that there was a decrease in enzyme activity under LSF, as compared to SSF. A decrease of 33.48% in LSF as compared to SSF for isolate A, a decrease of 31.35% in LSF as compared to SSF for isolate B, a decrease of 85.95% in LSF as compared to SSF for isolate C, a decrease of 54.25% in LSF as compared to SSF for isolate D, a decrease of 64.63% in LSF as compared to SSF for isolate E, a decrease of 76.49% in LSF as compared to SSF for isolate F, and a decrease of 56.98% in LSF as compared to SSF for isolate G, was observed. Thus, SSF was found to be the preferable mode of cultivation for all the test isolates. The greater xylanase production under SSF compared to LSF might be because SSF provided the fungus with an environment closer to its natural habitat (wood and decayed organic matter). This might have stimulated these strains to produce more hemicellulolytic enzymes [33]. Generally, in submerged cultivation, the growth form of filamentous fungi varies between pelleted and filamentous. Each form has its own characteristics and can affect the rate of enzyme production by influencing the mass transfer rate [33]. In Liquid State Fermentation (LSF), the fungus is exposed to hydrodynamic forces, while in SSF; growth is restricted to the surface of the solid matrix, with no such negative effects [32]. Also, the hyphal mode of growth gives the filamentous fungi, the power to penetrate into the solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution, as in the case of LSF. This makes the action of hydrolytic enzymes very efficient, and allows penetration into moist solid substrates. Penetration increases the accessibility of all the available nutrients within particles [34], and thus, enzyme production is higher in SSF. Also, catabolite repression and protein degradation by proteases that are severe problems in SmF, have often been reported to be reduced or absent in SSF [35].

As is evident from table 2, the isolate C showed the highest xylanase activity (697 IU/mL), after incubation period of 7 days, followed by isolates E (131.25 IU/mL)>D (94 IU/mL >B (80.41 IU/mL)>F (54.89 IU/mL)>G (17.90 IU/mL)>A (4.51 IU/mL), in that particular descending order, under conditions of SSF. The isolates showed poor cellulase activity and none of the isolates were found to be a non-cellulase producer. All the isolates also showed laccase production. Still the isolates can be employed for pulp biobleaching, as the cellulase activity reported was minimal in all the cases. Out of 7, 6 isolates (A, C, D, E, F, and G) showed maximum xylanase production after

Nutrient medium	Growth characteristics	Fungal Isolates	
		C	E
WBA	Growth (cm)	Full plate growth	Over growth
	Appearance	Creamy white, aerial	Creamy white, aerial
RBA	Growth (cm)	2.3	2.5
	Appearance	Creamish, sparse	Dull white, sparse
SBA	Growth (cm)	2.8	3.0
	Appearance	Creamy white, aerial, sparse	Creamy white, aerial, sparse
PDA	Growth (cm)	3.5	Full plate growth
	Appearance	Dull white, lateral	Dull white, lateral
SDA	Growth (cm)	0.8	2.1
	Appearance	Creamy white, lateral	Creamy white, aerial
XA	Growth (cm)	0.7	1.0
	Appearance	Dull white, irregular, sparse	Dull white, sparse, irregular
MEA	Growth (cm)	2.0	1.4
	Appearance	Dull white, aerial	Dull white, aerial

Temperature, °C=37
Incubation period, days=4

Table 3: Effect of composition of culture medium on the growth of test strains.

7 days of incubation, while 1 isolate (B) showed maximum xylanase production after 6 days of incubation. It is possible that other isolates might have different lag phase, and if the substrate was sampled later, a higher enzyme concentration might have been achieved. However, it was in our interest to compare the enzyme activities on same day, as short incubation periods are desirable from an industrial point of view. Though isolate B showed a shorter incubation period (shorter by 24 hrs) for maximum xylanase production, yet as compared to some of the isolates (isolate C, D, E) exhibiting maximum xylanase production after 7 days of incubation, it showed much lesser xylanase activity (88.46, 14.45 and 38.73% lesser xylanase activity, as compared to isolate C, D and E, respectively).

Effect of nutrient growth medium on the growth of fungal isolates

Two fungal isolates showing the best xylanase activities, with no/minimal cellulase activity were further grown on different agar media of defined (PDA, SDA, XA, MEA), and undefined (WBA, RBA, SBA) composition. This was done to observe the effect of nutrient medium on the growth of test isolates. As shown in table 3, both the isolates (C and E) showed the best growth on WBA, followed by PDA>SBA>RBA>MEA>SDA>XA, in that particular order. Though, PDA supported growth of the test strains, yet not better than WBA. The frequency of contamination by competing fungal species was also higher in case of PDA, as compared to WBA. This might be because WBA acts as a selective medium, and the many of the competing fungal species cannot easily utilize complex nutrients of wheat bran, which would be far easier in case of PDA; a defined medium containing simple sugars. The use of synthetic, defined media like PDA, MEA, SDA, and XA agar media is also not cost effective from the industrial point of view. Thus, WBA was chosen for cultivation of the test strains.

Conclusion

The indigenously isolated white rot fungal strain exhibited variable xylanase activity, with minimal cellulase activity. Two of the isolates (isolates C and E) produced good xylanase activity, minimal cellulase activity, and even exhibited laccase activity, thereby indicating that these white tor fungal strains can very well be tested as pulp biobleaching agents. All the isolates were found to exhibit better xylanase production under SSF, as compared to LSF. Wheat bran agar medium, a cheap growth medium, was found to be the best growth medium for isolates C and E. Thus, isolates C and E can be tested for xylanase production for pulp biobleaching in a cost effective manner.

Acknowledgement

Shalini Singh thanks the Ministry of Human Resource Development (MHRD), Government of India, for awarding her the senior research fellowship.

References

1. Kuhad RC, Singh A (1993) Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13: 151-172.
2. Wong KKY, Saddler JN (1992) *Trichoderma* xylanases, their properties and purification. Crit Rev Biotechnol 12: 413-435.
3. Roncero MB, Torres AL, Colom JF, Vidal T (2003) Effect of xylanase on ozone bleaching kinetics and properties of *Eucalyptus* kraft pulp. J Chem Technol Biotechnol 78: 1023-1031.
4. Viikari L, Kantelinen A, Sundquist J, Linko M (1994) Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Rev 13: 335-350.
5. Viikari L, Ranva M, Kantelinen A, Sandquist J, Linko M (1986) Bleaching with enzymes. Third International Conference in Biotechnology in Pulp and Paper Industry, Stockholm 67-69.
6. Tenkanen M, Viikari L, Buchert J (1997) Use of acid-tolerant xylanase for bleaching of kraft pulps. Biotechnology Techniques 11: 935-938.
7. Wong KKY, Saddler JN (1993) Applications of hemicellulases in the food, feed, and pulp and paper industries. In: Coughlan MP, Hazlewood GP (Eds.), Hemicelluloses and hemicellulases Portland Press, London 127-143.
8. Pawar S, Mathur RM, Kulkarni AG (2002) Control of AOX discharges in pulp and paper industry-the role of new fiberline Ippta Convention Issue 57.
9. Ragauskas AJ, Poll KM, Cesternino AJ (1994) Effects of xylanase pretreatment procedures on nonchlorine bleaching. Enzyme Microb Technol 16: 492-495.
10. Srinivasan MC, Rele MV (1999) Microbial xylanases for paper industry. Curr Sci 77:137-142.
11. Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit Rev Biotechnol 22: 33-64.
12. Techapun C, Poosaran N, Watanabe M, Sasaki K (2003) Thermostable and alkaline-tolerant microbial cellulase-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocess: a review. Process Biochem 38: 1327-1340.
13. Roncero MB, Torres AL, Colom JF, Vidal T (2000) Effects of xylanase treatment on fiber morphology in total chlorine free bleaching (TCF) of *Eucalyptus* pulp. Process Biochem 36: 45-50.
14. Viikari L, Suurnakki A, Buchert J (1996) Enzyme-aided bleaching of kraft pulps: fundamental mechanisms and practical applications. In: Jeffries TW, Viikari L, (Eds.), Proceedings of the ACS symposium series 655 on enzymes for pulp and paper processing, Washington DC: American Chemical Society 15-24.
15. Senior JS, Mayers PR, Saddler JN (1989) Production and purification of xylanases. In: Lewis NG and Paice MG (Eds.), Plant Cell Wall Polymers, Biogenesis and Biodegradation. ACS Symposium Series 399, American Chemical Society, Washington, DC 641-654
16. Qinnghe C, Xiaoyu Y, Tiangui N, Cheng J, Qiugang M (2004) The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. Process Biochem 39: 1561-1566.
17. Kirk TK, Connors WJ, Zeikus JG (1977) Advances in understanding the microbiological degradation of lignin. Recent Advances in Phytochemistry 11: 369-394.
18. Tenkanen M, Siika-aho M, Hausalo T, Puls J, Viikari L (1996) Synergism of xylanolytic enzymes of *Trichoderma reesei* in the degradation of acetyl-4-O-methylglucuronoxylan. In: Srebotnik E, Messener K (Eds.), Biotechnology in the pulp and paper industry Vienna: Facultas-Universitatsverlag 503-508.
19. Rosales E, Couto SR, Sanromán A (2002) New uses of food wastes: application to laccase production by *Trametes hirsuta*. Biotechnol Lett 24: 701-704.

20. Pellinen J, Abuhasan J, Joyce TW, Chang HM (1989) Biological delignification of pulp by *Phanerochaete chrysosporium*. Journal of Biotechnology 10: 161-170.

21. Addleman K, Archibald FS (1993) Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. Appl Environ Microbiol 59: 266-273.

22. 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: 777-780.

23. Singh SK, Garg AP (1996) Bioconversion of pea crop residues using *Gliocladium virens*. J Indian Bot Soc 75: 245-250.

24. Vishniac W, Santer M (1957) The Thiobacilli. Bacteriol Rev 21: 195-213.

25. Khandeparker R, Bhosle NB (2006) Isolation, purification and characterization of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation. Enzyme Microb Technol 39: 732-742.

26. Muthezhilan R, Ashok R, Jayalakshmi S (2007) Production and optimization of thermostable alkaline xylanase by *Penicillium oxalicum* in solid state fermentation. African Journal of Microbiology Research, Academic Journals 1: 20-28.

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

28. Mandels M (1975) Microbial sources of cellulases. Biotech Bioengg Symp 5: 81-105.

29. Ride JP (1980) The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. Physiol Plant Pathol 16: 187-192.

30. Sanghi A, Garg N, Sharma J, Kuhar K, Kuhad RC, et al. (2008) Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid-state fermentation. World J Microbiol Biotechnol 24: 633-640.

31. James TY, Srivilai P, Kües U, Vilgalys R (2006) Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics 172: 1877-1891.

32. da Silva R, Lago ES, Merheb CW, Macchione MM, Park YK, et al. (2005) Production of xylanase and CMCase on solid state fermentation in different residues by *Thermoascus aurantiacus* miehe. Braz J Microbiol 36: 235-241.

33. Mitchell DA, Lonsane BK (1992) Definition, characteristics and potential in solid substrate cultivation. In: Doelle HW, Mitchell DA, Rolz CE (Eds.), Solid State Fermentation, Elsevier, New York, Applied Science 455-467.

34. Raimbault M (1998) General and microbiological aspects of solid substrate fermentation. Electronic Journal of Biotechnology 1: 1-15.

35. Aguilar CN, Augur C, Favela-Torres E, Viniegra-González G (2001) Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid. J Ind Microbiol Biotechnol 26: 296-302.