

PRODUCTION OF NOVEL ALKALI-THERMO-TOLERANT CELLULASE-POOR XYLANASES FROM *COPRINOPSIS CINEREA* HK-1 NFCCI-2032

Harjeet Kaur,* Dharm Dutt, and C. H. Tyagi

Culture conditions of a newly isolated basidiomycetous strain were optimized for the enhanced production of extracellular alkali-thermo-tolerant cellulase-poor xylanase using wheat bran as the sole carbon source under solid state fermentation (SSF). SEM and ITS sequencing confirmed it as *Coprinopsis cinerea* HK-1 NFCCI-2032. Among various inexpensive agro-residues, wheat bran (carbon source) came up as the most potent enzyme inducer under SSF, and resulted in 54% higher xylanase activity compared to that in submerged fermentation mode. The strain grew well even at 47 °C. The highest xylanase (695.8 IU/mL) titer was recorded at a substrate:moisture ratio of 1:3 after 7 days of incubation at 37 °C at pH 6.4 along with 0.541 IU/mL of poorly associated cellulase activity. The xylanase exhibited remarkable stability and retained 50% of its activity at pH 8.0 on incubation at 55 °C for 15 min and 78, 43, and 23% of its activity at temperatures 65, 75 and 85 °C, respectively, demonstrating an approximately 50% alkali-thermo-tolerant nature, which is suitable for biobleaching.

Keywords: *Coprinopsis cinerea* HK-1 NFCCI-2032; ITS sequencing; Alkali-thermo-tolerant; Cellulase-poor xylanases; Solid-state fermentation

Contact information: Department of Paper Technology, Indian Institute of Technology Roorkee, Saharanpur campus, Saharanpur 247 001, India; *Corresponding author: dharm_dutt@yahoo.com, harjeet.iitr@gmail.com

INTRODUCTION

Xylanases have great potential in various industrial processes, including the manufacture of bread, food, and drinks, improvement of nutritional properties of agricultural silage and grain feed, for processing plant fibers in the textile industry, in pharmaceutical and chemical applications, and in the cellulose pulp and paper manufacturing processes (Beg et al. 2001). Xylan biodegradation is performed by a xylanolytic complex, which is primarily produced by fungi and bacteria. Xylanases (endo-1→4-β-xylanase, EC 3.2.1.8) are glycoside hydrolases catalyzing the endolytical hydrolysis of 1→4-β-D xylosidic linkages of the xylan backbone, a highly branched plant heteropolysaccharide (Polizeli et al. 2005; Poorna and Prema 2007). Hydrolysis of xylan is an important step towards appropriate utilization of lignocelluloses in nature. Chemical hydrolysis of lignocelluloses during bleaching has become an issue of great apprehension, primarily because of the hazardous by-products caused by the release of absorbable organic halides (AOX). These have prompted the increased use of microbial enzymes, which are specific in action for xylan hydrolysis and constitute a less capital intensive and more environmentally benign alternative (Biely 1985).

Because biotechnological applications necessitate large amounts of low-cost enzymes, an appropriate approach for such novel microbial isolates capable of producing cellulase-poor, alkali-thermo-tolerant xylanases for the paper industry is the utmost requirement, as the manufactured pulp after brown-stock washing has high temperature (about 70 °C) and pH (about 8.5). The plant species *Cymbopogon martini* (sofia grass), *Cymbopogon flexuosus* (lemon grass) (Kaur et al. 2011), *Saccharum officinerum*-CO 89003 (bagasse) (Agnihotri et al. 2010), *Triticum aestivum* PBW-343 (wheat straw) (Singh et al. 2011), and kraft AQ pulps of *Cannabis sativa* (true hemp), *Ipomea carnea* (besharam) (Dutt et al. 2008), *Hibiscus cannabiss* (kenaf) and *Hibiscus subdariffa* (roselle) (Dutt et al. 2009) have relatively open and loose fiber structure, which is suitable for enzyme action, and the pulp has more permeability than wood pulp. The pH after washing and screening ranges between 7.8 and 8.1. To make the enzyme applications more cost-effective at an industrial level, its production using cheap lignocellulosic wastes such as wheat straw, wheat bran, and corncob, rather than the more expensive xylan, has been recommended by many workers (Bokhari et al. 2010; Agnihotri et al. 2010; Gupta et al. 2001; Poorna and Prema 2007). One alternative for this is the use of solid state fermentation (SSF), which is closer to a natural system and advantageous in terms of higher productivity per reactor volume, lower operational and capital cost, as well as easier in terms of operation compared to conventional fermentation. It also has proved to be more efficient in producing certain enzymes and metabolites (Pandey et al. 2000; Agnihotri et al. 2010; Sanghi et al. 2008; Krishna 2005).

Until now the production of thermo-stable xylanases has been widely studied in submerged cultures, but the relatively high cost of enzyme production has hindered their industrial application. Two strains of *Coprinellus disseminates*, namely SH-1 and SH-2, show high xylanase activity (727.78 and 227.99 IU/mL) with very low CMC_{case} (0.925 and 0.660 IU/mL) and laccase (0.640 and 0.742 U/mL) activities at an incubation time of seven days, temperature 37 °C and initial pH of 6.4, using yeast extract as nitrogen source and wheat bran as cheap carbon source under SSF conditions. Crude cellulase-poor xylanases obtained from test strains show maximum activities at 55 °C and pH 6.4 (Shalini et al. 2009). The strain of *C. disseminatus* SW-1 NTCC 1165 produces high xylanase (499.60 IU/mL) and laccase activity (25.3 IU/ml) with minimum cellulose activity (0.64 IU/mL) when cultivated on media containing cheap agro-residue wheat bran as a sole carbon source and soya bean meal as nitrogen source on the 7th day of incubation at 37 °C and pH 6.4 under SSF conditions. Biochemical characterization reveals that optimal xylanase activity is observed at pH 6.4 and temperature 55 °C (Agnihotri et al. 2010).

The basidiomycete *Coprinopsis cinerea* has been a classic experimental model for multicellular development in fungi because it grows on defined media, completes its life cycle in 2 weeks, produces some 108 synchronized meicytes, and can be manipulated at all the developmental stages by mutation and transformation (Kües 2000). It is commonly isolated from horse dung (Kües 2000), degraded forestry products, or composting materials. Their occurrence may closely be associated with the ability to produce a variety of cell wall degrading enzymes. Xylanase production has been described for many fungal species, including the thermo-tolerant and thermophilic fungi (Ghatora et al. 2006), but until now, no study on the xylanase production from any *Coprinopsis cinerea*

strain using SSF has ever appeared in the literature. The present study was aimed at investigating the xylanase producing potential of indigenously isolated wild strain of *Coprinopsis cinerea* HK-1 NFCCI-2032, in context with its alkali-thermo-tolerant nature. The study examined a detailed optimization of SSF culture conditions, including pH, temperature, the selection of cheap and readily available carbon and nitrogen substrates, and the ratio of substrate to nutrient salt solution (NSS) for high production of xylanase. The biochemical characterization of enzyme was further made, marking the effect of pH and temperature on the activity and stability of xylanase. To the best of author's knowledge, this is the first paper that reports the production of xylanases by the basidiomycetous fungus *C. cinerea*.

MATERIALS AND METHODS

Isolation and Identification

The fungal strain was isolated by the enrichment technique from a rotten wood sample collected from the waste dumping site in the vicinity of Department of Paper Technology, Saharanpur, located in the foothills of Western UP (India). Isolation was carried out using wheat bran as a foremost carbon source, while maintaining a pH of 6.0 at 32 °C. The fungal isolate was maintained and routinely subcultured on wheat bran agar medium at pH 6.0. Purified cultures were transferred to potato dextrose agar (PDA) slants, incubated at 32 °C for 5 days, and further stored at 4 °C for future use. The molecular identification up to species level was done at the Agharkar Research Institute (ARI), Pune (India), by isolation of the genomic DNA in pure form, followed by amplification of nearly 3000 base pair rDNA fragments using 4 different pairs of universal primers (SSU-NS1 and NS4, NS3 and NS8, LSU-5.8SR and LR7, LR7R and LR12). The sequencing PCR was set up with ABI-BigDye® Terminator v3.1 Cycle Sequencing Kit (Part No. 4337455), and the sequence data was manually edited using appropriate software, whereby the test isolate was identified as *Coprinopsis cinerea* HK-1 with accession no NFCCI 2032.

Scanning Electron Microscopy (SEM)

The detailed morphological study of fungal strain HK-1 was carried out using SEM (SEM, Leo 435 VP, England). The fungal mat was fixed using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double-distilled water. The samples were then dehydrated under the alcohol gradients of 30, 50, 70, 80, 90, and 100% for 15 min each up to 70% alcohol gradient, and thereafter treated for 30 min under each gradient. After 100% alcohol treatment, samples were air dried and examined under SEM at desired magnifications.

Xylanase Production under Submerged (SmF) and Solid-state Fermentation

For production of extracellular enzymes, the nutrient salt solution (NSS) was prepared according to Singh and Garg (1995). The medium contained, in g/L, 1.5 KH₂PO₄, 4.0 NH₄Cl, 0.5 MgSO₄·7H₂O, 0.5 KCl, and 1.0 yeast extract in distilled water

with 0.04 mL/L trace elements solution having, in $\mu\text{g/L}$, 200 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 180 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 20 $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 6.0. SmF involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth. The main function of a fermenter is to provide a controlled environment for growth of microorganisms in order to obtain a desired product. Two important criteria for a submerged liquid fermenter include the ability to operate aseptically for a number of days and provide adequate aeration and agitation to meet the metabolic requirements of the microorganism. SmF was carried out in 40 mL of NSS containing 2% of wheat bran fraction (100 μm mesh size) as a carbon source in an Erlenmeyer flask (250 mL). Culture medium was autoclaved at 15 psi for 15 min, inoculated with fungal isolate HK-1, and incubated in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking at 120 rpm. Solid substrate fermentations are generally characterized by growth of microorganisms on water-insoluble substrates in the presence of varying amounts of free water (Mitchell and Lonsane, 1992). For SSF, after addition of NSS (15 mL) to wheat bran (5 g), the flasks, covered with cotton plugs and aluminium foil, were autoclaved at 121°C and 15 psi for 15 min. On cooling, flasks were inoculated with 2 discs, each of 5 mm diameters cut from the periphery of six-day-old culture and incubated at 32 °C.

Enzyme Harvesting and Storage

In SmF, enzyme was filtered through four-layered muslin cloth, while in SSF the contents of the flask were crushed with a glass rod using 15 mL of distilled water and were shaken for 30 min to harvest the enzyme from the fungal cells. The whole content was then filtered through the four layers of muslin cloth, and the filtrate so obtained was centrifuged (Sigma centrifuge model 2K15) at 5000 g for 10 min at 4 °C. The clear brown coloured supernatant was used as crude enzyme extract and stored at -20 °C until use.

Estimation of Enzyme Activity and Protein Concentration

Xylanase activity was estimated by analysis of the xylose released by the DNS method (Miller 1959). For this purpose, 0.4 mL of 1% birch wood xylan solution was mixed with 1.6 mL of suitably diluted culture filtrate in 50 mM K_3PO_4 buffer (pH 6.4) and incubated at 55 °C for 15 min. A 0.3 mL of solution was taken from the incubated mixture in a test tube, and 0.9 mL of dinitrosalicylic acid (DNS) reagent was added and boiled for 5 min. Xylose released was estimated colorimetrically at 540 nm using a UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 °C. Carboxymethyl-cellulase (CMCase) activity was determined according to the method of Mandel (1975). The assay mixture, in a total volume of 2 mL, contained 0.5 mL of 1 mM of CMC in 50 mM citrate buffer (pH 4.8) and 0.5 mL of the enzyme. The mixture was incubated at 50 °C for 30 min. The reducing sugars released in the hydrolysis reaction were measured optically at 575 nm by the DNS method as described by Miller (1959). Enzyme activities (xylanase and cellulase) were expressed as IU equivalent to micromoles of xylose or glucose released per min per mL of the reaction mixture under standard conditions.

Laccase assay (de Souza-Cruz, 2004) was carried out by taking 100 mM citric acid buffer at pH 5.0, enzyme extract, and 1.0 mM of ABTS (2, 2-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) as a substrate, and reaction was monitored at 420 nm. The

enzyme activity was expressed as the amount of enzyme that produced an increase of 1.0 absorbance unit per 30s. Protein concentration was determined as described by Lowry et al. (1951), with bovine serum albumin (BSA) as a standard. Controls were routinely included in which enzyme or substrate was omitted and treated similarly.

Optimization of Incubation Period, pH and Temperature

Culture conditions were optimized by varying one independent variable at a time while keeping the other variables constant. A set of Erlenmeyer flasks (250 mL) containing 5 g of wheat bran and 15 mL of NSS, was autoclaved and inoculated aseptically with fungal strain HK-1. These were incubated at 32 °C, pH 6.0, and harvested every day from the 2nd to the 11th day of incubation. Likewise, the initial pH of NSS for xylanase production was optimized by varying pH within 4.4 to 11, adjusted with 1N NaOH/H₂SO₄ separately by incubating inoculated flasks at 32 °C for seven days. Similarly, temperature variability regarding fungal xylanase production was carried out by incubating the inoculated flasks at temperatures ranging from 27 to 52 °C with an interval of 5 °C at optimum pH and incubation period. In each case, xylanase and cellulase activities were determined as described above.

Effect of Carbon and Nitrogen Sources and Moisture Level

The effects of lignocellulosic substrates (wheat straw, wheat bran, sugarcane bagasse, sorghum, and rice straw), singly and in different combinations, on xylanase production were studied. Five g each of finely powered lignocellulosic substrate retained on +100 mesh size and moistened with 15 mL of NSS was added in different flasks, and inoculated flasks (as above) were incubated at the conditions mentioned above. In the same way, different nitrogen sources (peptone, beef extract, yeast extract, malt extract, soya bean meal, and urea) in the NSS at a concentration of 1.0 g/L were inoculated with the test fungus and incubated at optimum incubation temperature, period, and pH. The effect of moisture level on the xylanase titre was evaluated by varying the wheat bran to NSS ratio (w/v) between 1:2, 1:2.5, 1:3, 1:3.5, and 1:4.0. The prepared flasks were incubated at pH 6.4 for first 7 days at 37 °C. Xylanase and cellulase activities were determined in each case.

Effect of Glucose and Lactose Concentration

The incubation medium was supplemented with 1 to 5 g/L of glucose/lactose in NSS solution while maintaining the wheat bran to NSS ratio at 1:3. The flasks were inoculated and incubated under the conditions mentioned above, and the effect of glucose/lactose was studied on xylanase titre.

Effect of pH and Temperature on Crude Xylanase Stability

The pH stability of crude xylanases was determined in the range of 6.0 to 9.0 by incubating the xylanase in buffers of different pH (potassium-phosphate; pH range: 6.0 to 7.4 and borax-boric acid; pH range: 7.6 to 9.0) for 15 min. For their thermo-stability, crude enzyme preparations were incubated at 45, 55, 65, 75, and 85 °C at pH 6.4 for 15 min. The residual xylanase activity (Miller 1959) of the crude enzyme in each case was determined under standard assay conditions as described above.

Statistical Analysis

All the experiments were conducted in triplicate, and experimental results were represented as the mean \pm standard deviation of three identical values.

RESULTS AND DISCUSSION

Isolation, Screening for Xylanase Production and SEM

The fungal isolate HK-1 was initially screened for its xylanase production ability by the xylan-agar (XA) plate assay. XA-plate was stained with Congo red dye to enhance the visibility of hydrolyzed area observed as a clear zone. The isolate was further subjected to a secondary screening for its xylanase production ability under SSF conditions (Fig. 1a). The strain produced 456.12IU/mL xylanase activity with a meagre cellulase activity of 0.744IU/mL using wheat bran as a sole carbon source at 32 °C for 6 days. The fungal isolate was hence selected for further studies based on the characteristic of providing high xylanase with poor-cellulase activity.

Morphological studies of the fungal isolate *C. cinerea* HK-1 with SEM revealed a spreading, white colored mycelial network of highly tapered, tubular, branched filamentous hyphae (Plate1a). These filaments exude enzymes and absorb food at their growing tips. Hyphae are collectively very long, and can explore and exploit food substrates very efficiently (Alexopoulos et al. 1996). Small etiolated stripes, arising after 4 to 5 days of inoculation, eventually grew into unique and complex structures called fruiting bodies, which subsequently suffered rapid autolysis, a feature that is specific for many species of the coprini. Black, club-shaped spores were produced by fruiting bodies on the 8th or 9th day (Plate1b). The identification was further confirmed by sequencing the SSU (SSU- NS1, NS4, NS3, NS8) and LSU region (LSU- LR7, LR7R, LR12) of rDNA for *C. cinerea* HK-1.

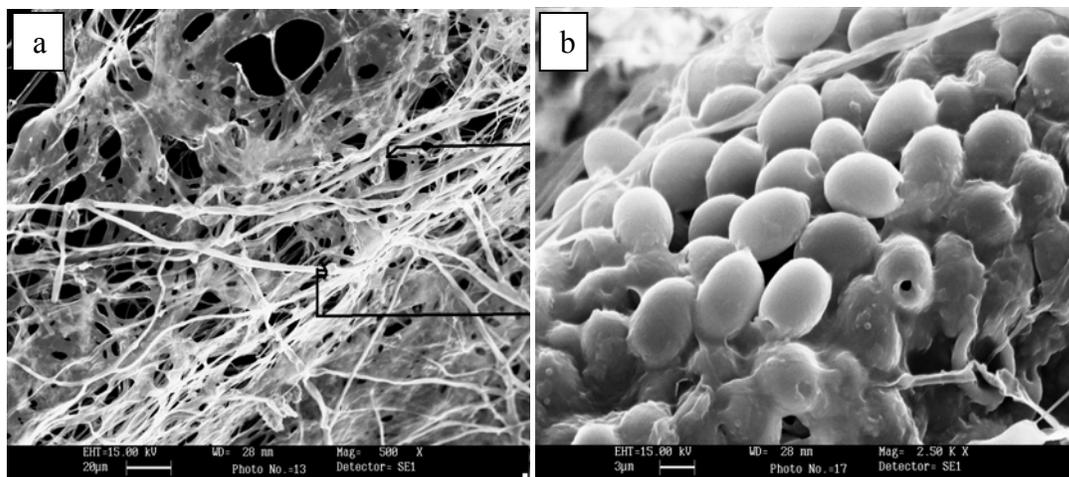


Plate 1. SEM microphotographs of *Coprinopsis cinerea* HK-1 agar showing fungal mycelia and clamp connection (arrows) (a), and mature club shaped spores (b)

Effect of Different Fermentation Conditions

Figure 1a reveals that the xylanase activity of *C. cinerea* HK-1 under SSF conditions was 54% higher to that in SmF. SSF aimed at strengthening the contact between fungal mycelia and the insoluble substrate, thereby achieving the highest substrate concentration (Hölker et al. 2004), which is not possible during SmF. Therefore, SSF was chosen for further optimization studies for crude xylanase production by the *C. cinerea* HK-1. A hike in xylanase production of *Coprinellus disseminatus* SW-1 NTCC1165 under SSF using wheat bran was reported by Agnihotri et al. (2010) in comparison to SmF, while a 30-fold enhancement in xylanase production under SSF was observed by Malarvizhi et al. (2003) in contrast to liquid culture using wheat bran as the substrate for a culture of *Ganoderma lucidum*.

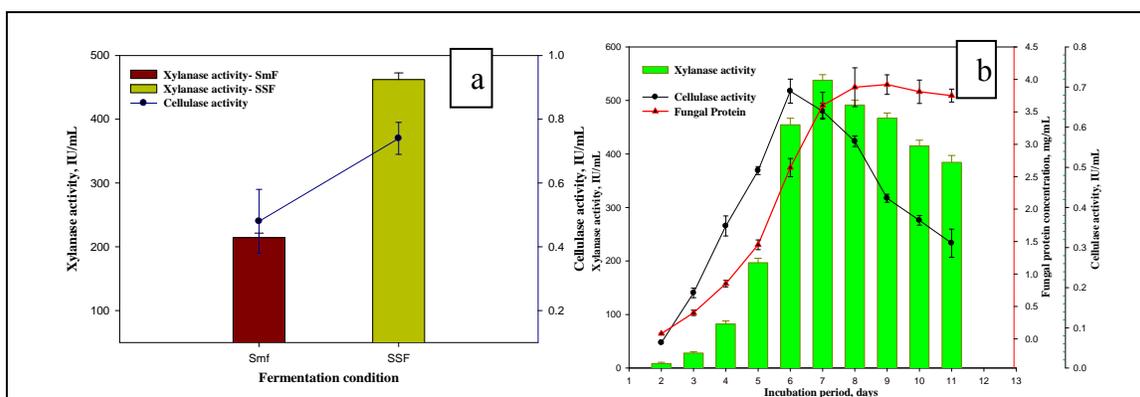


Fig. 1. Effect of different fermentation systems (a) and incubation period (b) on the enzyme production by *Coprinopsis cinerea* HK-1

Effect of Incubation Period

The xylanase production steadily attained its maxima with a 13.5% increase on the 7th day compared to the activity on 6th day at 32 °C and pH 6.4, while maximum cellulase production (0.690 IU/mL) was achieved on the 6th day of incubation (Fig. 1b). In some fungi, high xylanase production has been shown to be correlated firmly to cellulase production (Kang et al. 2004), but *C. cinerea* HK-1 did not produce much cellulase despite of the use of cellulose-rich substrate. Figure 1b indicates that the maximum xylanase activity (537.83 IU/mL) and protein biomass (3.92 mg/mL) achieved for the strain HK-1 almost corresponded to the optimized period of incubation. Basidiomycetes are slow-growing fungi, so maximum xylanase production was achieved on 7th day in *Pleurotus ostreatus* (Qinnghe et al. 2004). Xylanase production and supernatant protein concentration increased up to the 7th day of incubation for the two strains SH-1 and SH-2 of *C. disseminatus* (Singh et al. 2010). A reduction in xylanase titre (491.4IU/mL) obtained after optimum period was probably due to the depletion of nutrients available to the fungus. Xylanases being primary metabolites are optimally expressed at the end of the exponential phase, which correlates the harvesting time of the fungus to the production of enzymes (Kulkarni et al. 1999). Metabolic enzymes like, proteases and transglycosidases might also affect xylanase yield.

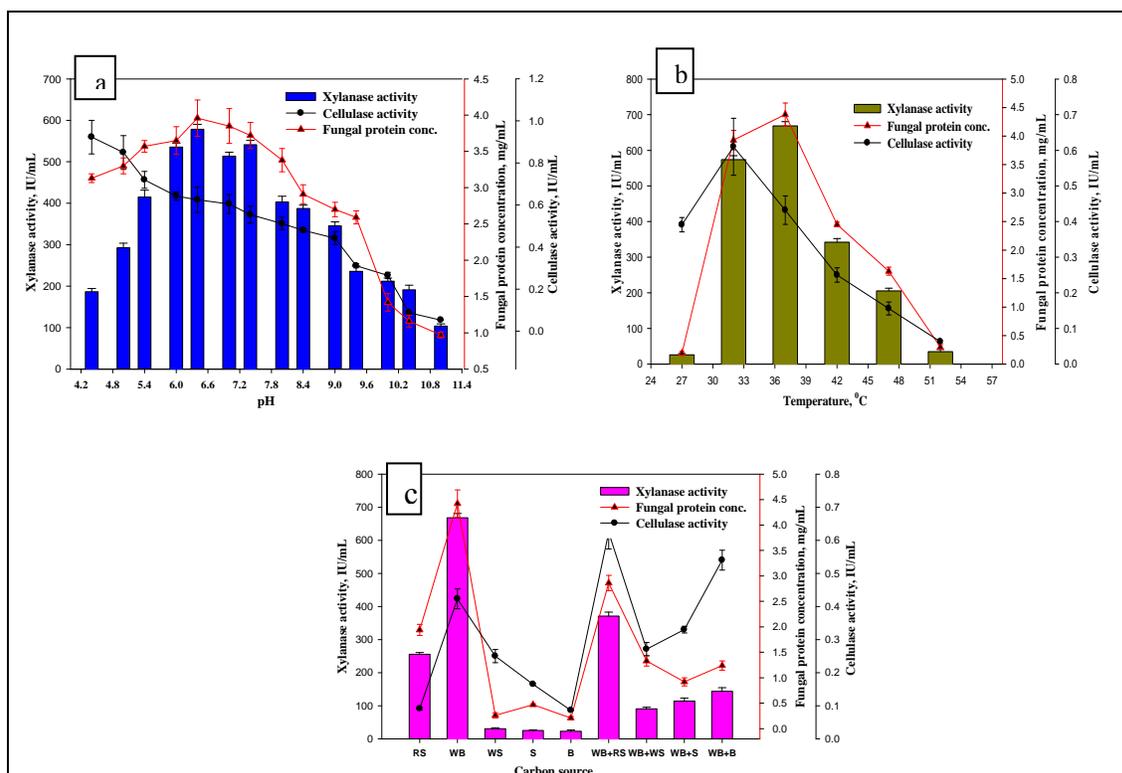


Fig. 2. (a) Effect of pH, (b) temperature and (c) lignocellulosic substrates on xylanase production by *Coprinopsis cinerea* HK-1

Effect of Initial pH

A pH of 6.4 was found to be optimal for fungal growth, with 578.27 IU/mL of xylanase activity. In addition, when NSS with pH between 6.0 and 7.4 were used, no significant differences were found in the enzyme yield (Fig. 2a). This could be explained by the fact that wheat bran possesses excellent buffering capacity (Pandey et al. 2000). Similar observations were made by Pal and Khanum (2010) for xylanase from *Aspergillus niger* DFR-5 in wheat bran-based medium. At a pH of 8.0, 70% of the enzyme activity was retained, which was quite remarkable. The enzyme production was severely hampered above an alkaline pH of 9.4 (235.80 IU/mL). Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (Poorna and Prema 2007). In view of the fact that enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are liable to be influenced by pH changes, and the catalytic property of the enzyme is strikingly affected. Fermentation at lower and higher pH proved to be detrimental, perhaps because of the inactivation of the enzyme system. Each microorganism thereby holds a certain pH range for its optimal growth and activity. Most of the white-rot fungi have been reported to grow best at slightly acidic pH (Reid et al. 1989). The optimum initial medium pH was 6.5 for xylanase production under shake flask fermentation condition using corncob and corn steep liquor as the carbon and nitrogen sources for both *T. lanuginosus* wild type and M7 (Bokhari et al. 2010), while, Ninawe et al. (2008) worked with purified xylanase from *Streptomyces cyaneus* SN at a pH 6.0.

Optimization of Incubation Temperature

Temperature is one of the important parameters that determine the success of the optimization system. *C. cinerea* HK-1 was found to grow well in a temperature range of 32 to 47 °C, and maximal xylanase titer (668.45 IU/mL) was obtained at 37 °C at pH 6.4 and incubation time 7 days. It retained 50% of its maximal production at 47 °C (Fig. 2b). An identical optimum temperature for xylanase production in SSF was reported by Beg et al. (2001) and Sanghi et al (2008). *S. cuspidosporus* also grew rapidly and produced maximum xylanase at a temperature of 37 °C (Maheswari and Chandra 2000).

A decrease in xylanase titer was obtained with cultivation temperatures either below or above the temperature optima. The decreased yield at low temperatures was possibly due to lower transport of substrates across the cells. On proceeding towards optimum temperature for enzyme production, increased kinetic energy of reacting molecules increase the reaction rate. At higher temperatures, thermal denaturation of enzymes of the metabolic pathway occurs, which increases the maintenance energy requirement of cellular growth, thereby resulting in poorer production of the metabolites (Aiba et al. 1973) and even loss of enzyme activity (Pal and Khanum 2010) signifying that the end-point of fermentation should be carefully controlled. It also has been stated that microorganisms synthesize only a reduced number of proteins essential for growth and other physiological processes under conditions of high temperatures (Gawande and Kamat 1999).

Selection of Solid Substrates for Xylanase Production in SSF

Requirements for efficient xylanase production differ from one fungal strain to another as far as carbon source requirement is concerned. Just providing nutrient to the microorganisms could not be a sufficient criterion for choosing a substrate; oxygen transfer and heat dispersion are equally important. The highest xylanase production for *C. cinerea* HK-1 (668.1IU/mL) was achieved when wheat bran was used for fermentation (Fig. 2c). Wheat bran is a suitable supporter and carrier because of its porosity and low cost. Wheat bran contains a blend of soluble sugars vital for the initiation of growth and replication of the microorganism. It contains 45% hemicellulose (40% xylans), which may fulfil the role of inducers, and organic nitrogen sources (23%) that are crucial for protein synthesis. Moreover, it provides a large surface area and efficient aeration by remaining loose even under moist conditions during the SSF mode of culturing (Babu and Satyanarayana 1995). Wheat bran has been described as a potent substrate and an enhancer for xylanase production in SSF by *Arthrobacter* sp., and *Streptomyces cyaneus* SN 32 (Ninawe et al. 2008).

Rice straw supported the fungal growth and xylanase production at the second highest level, with xylanase activities of 255.64 IU/mL. Wheat bran and rice straw together (1:1) worked out to be the best among all combinations, with the xylanase activity as 370.82 IU/mL, which was 31% more than that found by using rice straw individually. Recent research established a synergy between various inducers present in rice straw and wheat bran, and resulted in high production of cellulases and hemicellulases (Kaur et al. 2006). *C. cinerea* HK-1 reacted in an unexpectedly better manner under these combined carbon sources, with increasing xylanase activities in the

order wheat bran + rice straw > wheat bran + wheat straw > wheat bran + bagasse > wheat bran + sorghum.

Effect of Nitrogen Sources

The mechanisms that preside over the mycelial growth and formation of extra-cellular enzymes are influenced by the availability of precursors for protein synthesis (Kulkarni et al. 1999). Among the various complex organic nitrogen sources used (Fig. 3a), the order of suitability for production of xylanase was: beef extract > yeast extract > urea > malt extract > peptone > soya bean meal. Beef extract favored maximum xylanase productivity (690.71 IU/mL), which may be attributed to the better absorption of amino acids present in beef extract by the mycelia of the strain HK-1 (Qinnghe 2004). Soya bean meal as a source of nitrogen has been mentioned for yet another white-rot *Coprinellus disseminatus* SW-1 NTCC1165 (Agnihotri et al. 2010) under SSF; however, in the present study, xylanase production obtained with beef extract (690.71 IU/mL) increased by 16% in comparison to soya bean meal (583.34 IU/mL). These results indicate that the quality of proteins for enzyme production varies from species to species. Peptone was the best nitrogen source with white-rot basidiomycetes, to achieve optimal xylanase activity from *Pleurotus ostreatus* (Qinnghe. 2004) under SmF conditions and *Lentinus edodes* IBB 363 (Kachlishvili. 2005) under SSF.

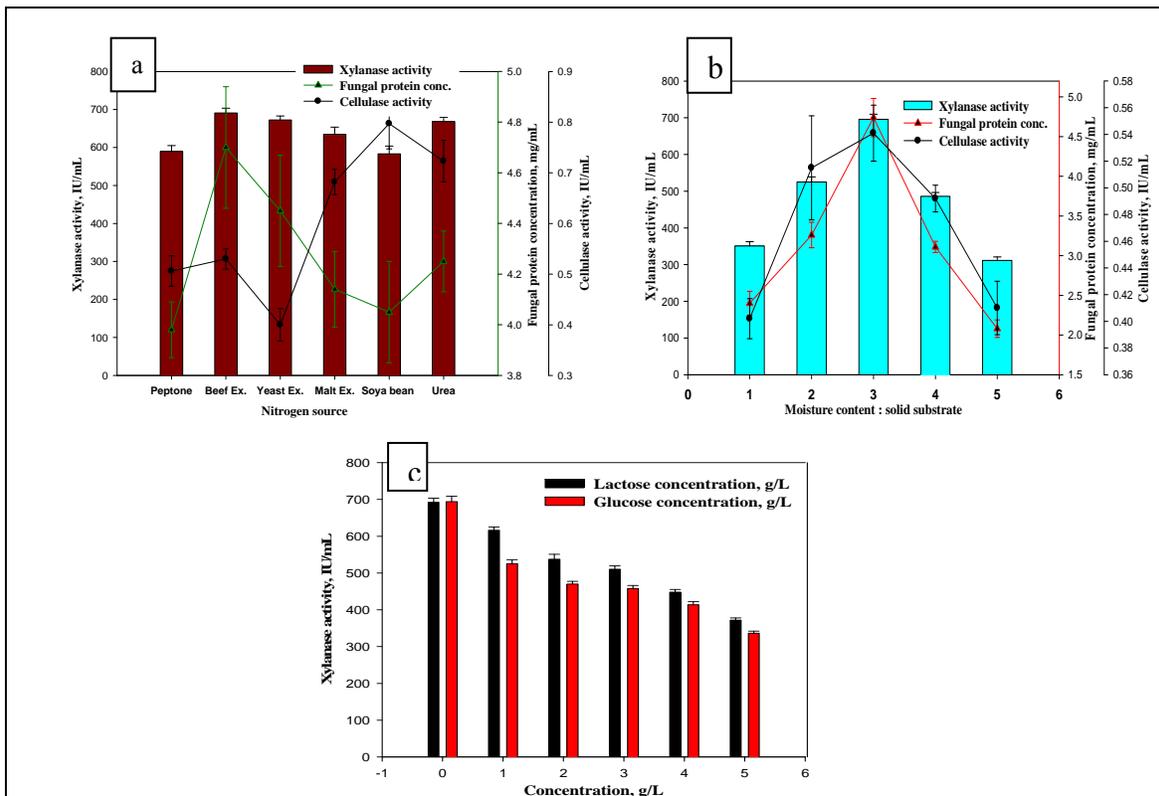


Fig. 3. Effect of (a) various nitrogen sources (b) moisture content and (c) simple sugars on xylanase production by *Coprinopsis cinerea* HK-1

Effect of Moisture Level

The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis might be attributed to the impact of moisture on the physical properties of the solid substrate. The optimal xylanase production (695.8 IU/mL) was obtained at a substrate to moisture ratio of 1:3 (Fig. 3b). By decreasing the ratio to 1:2, nearly half of the activity could be obtained. Nearly 45% of the maximum xylanase yield (311.2 IU/mL) was retained at a ratio of 1:4. An increase in the moisture content was found to be more detrimental as compared to a decrease. Under SSF conditions, the impact of moisture on the physical properties of the solid substrate influences the microbial growth and product biosynthesis. A higher than optimum moisture level caused a decrease in porosity, lower oxygen transfer, a gummy texture, and alteration in wheat bran particle structure, whereas a low moisture level resulted in reduced solubility of nutrients in the solid substrate and decreased swelling of the solid substrate. Maximum xylanase yield for *Streptomyces sp.* QG-11-3 was reported at the substrate to moisture ratio of 1:2.5 and 1:3 using wheat bran and eucalyptus pulp as the respective substrates (Beg et al. 2001).

Effect of Glucose and Lactose on Xylanase Production

The production of xylanase by *C. cinerea* HK-1 was found to be repressed by increasing concentrations of both glucose and lactose from 1 to 5 g/L, the maximum production being attained in the absence of these simple sugars (Fig. 3c). The production of extra-cellular xylanase by *C. cinerea* HK-1 was thereby inducible and controlled by catabolite repression. Glucose in fermentation medium generally represses enzyme production by moulds, and only after exhaustion of glucose, the fungus starts the production of xylanase. Such xylanase repression seems to be an indicative of the fact that enzyme synthesis is controlled by transition state regulators and catabolite repression as was also observed by Sanghi et al. (2008) in *Bacillus subtilis* ASH, using wheat bran under SSF at temperature 37 °C and pH 7.0 and Smith and Wood (1991) in *Aspergillus awamori* using ball-milled oat straw under SmF at temperature 30 °C and pH 4.0. On the contrary, resistance to catabolite repression by glucose has been cited from a mutant strain, M7 of *Thermomyces lanuginosus* (Bokhari et al. 2010).

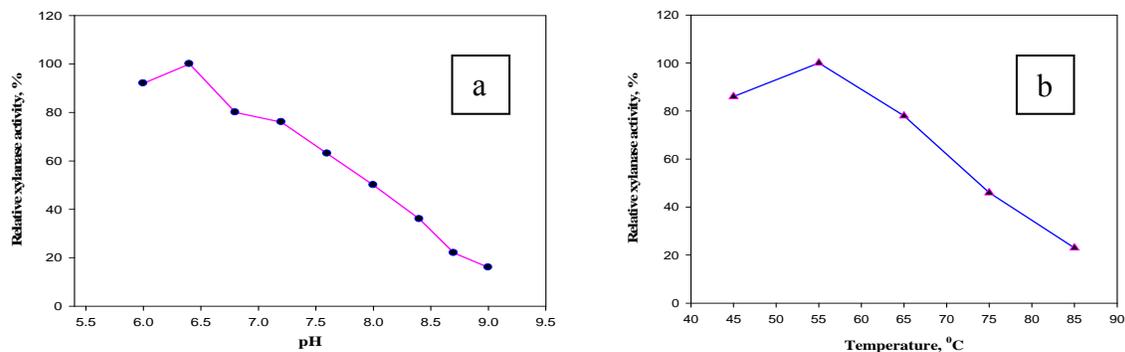


Fig. 4. Relative pH (a) & thermal tolerance (b) of crude xylanase from *Coprinopsis cinerea* HK-1

Effect of pH and Temperature on the Activity and Stability of Xylanase

The tolerance of alkaline conditions is crucial, especially when considering the potentiality of xylanases in bleaching of cellulose pulps at high pH. The crude xylanase extracted from *C. cinerea* HK-1 was most active in the pH range of 6.0 to 6.8, when incubated at 55 °C for 15 min and retained 50% of its activity at pH 8 (Fig. 4a), thereby proving its 50% alkali-tolerant nature. Enzymes, proteinaceous entities are liable to denaturation under harsh conditions posed by pH change, high temperature, or presence of high concentrations of metal ions, with adverse effects on their active sites resulting in a subsequent loss of enzyme activity. The pH activity profiles of enzymes also depend on pKa (ionization constant) of catalytic residues. The lower the pKa value, the higher is the pH stability. Amino acid residues contributing positive charges and hydrogen bonds lower the pKa values, with shorter bonds having a more definite effect (Collins et al. 2005). The optimal pH range for some strains of *T. lanuginosus* was found to be 6.0 to 7.0 (Singh et al. 2003). The activity of extracellular xylanase from *C. disseminatus* SW-1 NTCC1165 was found within a pH range of 6.0 to 9.0 with the optimum at pH 6.4 (Agnihotri et al. 2010). The thermal stability of the crude xylanase from *C. cinerea* HK-1 was examined within a temperature range of 45 to 85 °C at pH 6.4 for 15 min, and the results (Fig. 4b) are indicative of good thermal-tolerance. The xylanase retained 78% of its original activity at a temperature of 65 °C. Although incubation at further higher temperatures inactivated the enzyme, still nearly 46% and 23% activities were retained at temperatures of 75 and 85 °C, respectively. Similarly to the xylanase in this study, most of the other known xylanases are also optimally active in the range of 50 to 65 °C (Kulkarni et al. 1999).

Enzyme Production under Optimized SSF Conditions

Under optimum SSF conditions, xylanase activity was 693.6 IU/mL, laccase activity 26.5 U/mL, and supernatant protein concentration 4.8 mg/mL, for *C. cinerea* HK-1. Cellulase contamination in the crude enzyme extract decreased to 0.550 IU/mL after the optimization of temperature from 32 to 37 °C. Although an overestimation of soluble proteins in crude extracts because of interferences with other compounds was possible, its profile agreed with an increase in enzyme activities.

CONCLUSIONS

The present work established the potential of a newly isolated white-rot basidiomycete *C. cinerea* HK-1 NFCCI-2032 for maximum xylanase production. Solid state fermentation (SSF) was shown to be the preferred mode of enzyme production by the strain *C. cinerea*, producing 693.6 IU/mL extra cellular xylanase and 26.5 U/mL laccase with supernatant protein concentration (4.8 mg/mL) when cultivated on media containing cheap agro-residue wheat bran as a sole carbon source and beef extract as a nitrogen source on the 7th day of incubation at 37 °C and pH 6.4. Although an overestimation of soluble proteins in crude extracts because of interferences with other compounds was possible, its profile agreed with an increase in enzyme activities. The crude xylanase obtained from the SSF was associated with a negligible cellulase

contamination (0.55 IU/mL) under optimized conditions for xylanase production and so did not need any purification step. Thus it can be directly used in pulp and paper industry applications, which further reduce its cost. The removal of xylan along with lignin-carbohydrates complexes (LCC) during pulp prebleaching affects fibre swelling during pulp beating and development of cellulose-hemicelluloses hydrogen bonds adversely. A negligible quantity of cellulase causes hydrolysis of β -cellulose (degraded cellulose), which deposits on fiber surfaces and improves all the strength properties depending upon hydrogen bonding (tensile and burst indices and double fold numbers, etc.). In addition the xylanase was found to have a 50% thermo-alkali-tolerant nature, which is a typical prerequisite for pulp and paper industry applications. Considering the characteristics of *C. cinerea* HK-1 NFCCI-2032, it has a potential role in the development of a biobleaching process for mass production of xylanase, using low-cost media (wheat bran) and for commercial application in the paper industry.

ACKNOWLEDGMENTS

This work was supported by a grant from the Council of Scientific and Industrial Research (CSIR), New Delhi, Government of India, as a part of fellowship (SRF) for PhD. studies at IIT-Roorkee, Department of Paper Technology.

REFERENCES CITED

- Aiba, S., Humphrey, A. E., and Millis, N. F. (1973). "Kinetics," In: *Biochemical Engineering*, 2nd Edn., Academic Press, New York, pp. 92-127.
- Alexopoulos, C. J., Mims, C. W., and Blackwell, M. (1996). "Order Agaricales: The mushrooms," In: *Introductory mycology*, Alexopoulos, C. J., and Mims, C.W., and Blackwell, M. (eds.), John Wiley & Sons, New York, pp. 508-543.
- Agnohotri, S., Dutt, D., and Tyagi, C. H. (2010). "Complete characterization of bagasse of early species of *Saccharum officinerun*-Co 89003 for pulp and paper making," *BioResources* 5(2), 1197-1224.
- Agnihotri, S., Dutt, D., Tyagi, C. H., Kumar, A., and Upadhyaya, J. S. (2010). "Production and biochemical characterization of a novel cellulase-poor alkali-thermo-tolerant xylanase from *Coprinellus disseminatus* SW-1 NTCC1165," *World J. Microbiol. Biotechnol.* 26, 1349-1359.
- Babu, K. R., and Satyanarayana, T. (1995). " α -Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation," *Process Biochem.* 30, 305-309.
- Battan, B., Sharma, J., Dhiman, S. S., and Kuhad, R. C. (2007). "Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry," *Enzyme Microbial Technol.* 41, 733-739.
- Beg, Q. K., Kapoor, M., Mahajan, L., and Hoondal, G. S. (2001). "Microbial xylanase and their industrial application: A review," *Appl. Microbiol. Biotechnol.* 56, 326-338.

- Biely, P., Mislovicová, D., and Toman, R. (1985). "Soluble chromogenic substrates for the assay of endo 1,4- β -xylanases and endo 1,4- β -glucanases," *Anal. Biochem.* 144(1), 142-146.
- Bokhari, S. A. I., Rajoka, M. I., Javaid, A., Shafiq-ur-Rehman, Ishtiaq-ur-Rehman, and Latif, F. (2010). "Novel thermodynamics of xylanase formation by a 2-deoxy-D-glucose resistant mutant of *Thermomyces lanuginosus* and its xylanase potential for biobleachability," *Bioresour. Technol.* 101, 2800-2808.
- Collins, T., Gerday, C., and Feller, G. (2005). "Xylanases, xylanase families and extremophilic xylanases," *FEMS Microbiol. Rev.* 29, 3-23.
- de Souza-Cruz, P. B., Freer, J., Siika-Aho, M., and Ferraz, A. (2004). "Extraction and determination of enzymes produced by *Ceriporiopsis subvermispota* during biopulping of *Pinus taeda* wood chips," *Enzyme Microbial. Technol.* 34, 228-234.
- Dutt, D., Upadhyay, J.S., Tyagi, C. H., and Kumar, A. (2008). "Studies on *Ipomea carnea* and *Cannabis sativa* as an alternative pulp blend for softwood: An optimization of kraft delignification process," *Ind. Crops Prod.* 28, 128-136.
- Dutt, D., Upadhyay, J. S., Singh, B., and Tyagi, C. H. (2009). "Studies on *Hibiscus cannabinus* and *Hibiscus sabdariffa* as an alternative pulp blend for softwood: An optimization of kraft delignification process," *Ind. Crops Prod.* 29, 16-26.
- Gawande, P. V., and Kamat, M. Y. (1999). "Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application," *J. Appl. Microbiol.* 87, 511-519.
- Gupta, S., Kuhad, R. C., Bhushan, B., and Hoondal, G. S. (2001). "Improved xylanase production from a haloalkalophilic *Staphylococcus* sp. SG-13 using inexpensive agricultural residues," *World J. Microbiol. Biotechnol.* 17, 5-8.
- Hölker, U., Höfer, M., and Lenz, J. (2004). "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi," *Appl. Microbiol. Biotechnol.* 64, 175-186.
- Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., and Kim, S. W. (2004). "Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass," *Biores. Technol.* 91, 153-156.
- Kachlishvili, E., Penninckx, M.J., Tsiklauri, N., and Elisashvili, V. (2005). "Effect of nitrogen source on lignocellulosic enzyme production by white-rot basidiomycetes under solid-state cultivation," *World J. Microbiol. Biototechnol.* 22(4), 391-397.
- Kaur, J., Chadha, B. S., and Saini, H. S. (2006). "Regulation of cellulase production in two thermophilic fungi *Melanocarpus* sp. MTCC 3922 and *Scytalidium thermophilum* MTCC 4520," *Enzyme Microbial. Technol.* 38, 931-936.
- Kaur, H., Dutt, D., and Tyagi, C. H. (2011). "Optimization of soda pulping process of ligno-cellulosic residues of lemon and sofia grasses produced after steam distillation," *BioResources* 6(1), 103-120.
- Krishna, C. (2005). "Solid-state fermentation systems – An overview," *Crit. Rev. Biotechnol.* 25, 1-30.
- Kües, U. (2000). "Life history and developmental processes in the basidiomycete *Coprinus cinereus*," *Microbiol. Mol. Biol. Rev.* 64, 316-353.
- Kulkarni, N., Abhay, S., and Rao, M. (1999). "Molecular and biotechnological aspects of xylanases," *FEMS Microbiol. Rev.* 23, 411-456.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). "Protein measurement with the folin phenol reagent," *J. Biol. Chem.* 193, 265-275.
- Maheswari, M. U., and Chandra, T. S. (2000). "Production and potential applications of a xylanase from a new strain of *Streptomyces cuspidosporus*," *World J. Microbiol. Biotechnol.* 16, 257-263.
- Malarvizhi, K., Murugesan, K., and Kalaichelvan, P. T. (2003). "Xylanase production by *Ganoderma lucidum* on liquid and solid-state culture," *Indian J. Exp. Biol.* 41, 620-626.
- Mandels, M., Andreotti, R., and Roche, C. (1976). "Measurement of saccharifying cellulose," *Biotechnol. Bioeng. Symp.* 6, 21-23.
- Miller, G.L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugars," *Anal. Chem.* 31, 426-428.
- Mitchell, D.A., and B.K. Lonsane. (1992). "Definition, characteristics and potential," In: *Solid Substrate Cultivation*, Doelle, H. W., Mitchell, D. A., and Rolz, C. E. (eds.), Elsevier Applied Biotechnology Series.
- Ninawe, S., Kapoor, M., and Kuhad, R. C. (2008). "Purification and characterization of extracellular xylanase from *Streptomyces cyaneus* SN32," *Bioresour. Technol.* 99, 1252-1258.
- Pal, A., and Khanum, F. (2010). "Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation," *Biores. Technol.* 101, 7563-7569.
- Pandey, A., Socool, C. R., and Mitichell, D. (2000). "New developments in solid-state fermentation," *Process Biochem.* 35, 1153-1169.
- Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., and Amorim, D. S. (2005). "Xylanases from fungi: Properties and industrial applications," *Appl. Microbiol. Biotechnol.* 67, 577-591.
- Poorna, C. A., and Prema, P. (2007). "Production of cellulase-free endoxylanase from novel alkalophilic thermotolerant *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling," *Bioresour. Technol.* 98, 485-490.
- Quinnge, C., Xiaoyu, Y., Tianguai, N., Cheng, J., and Qiugang, M. (2004). "The screening of culture conditions and properties of xylanase by white-rot fungi *Pleurotus ostreatus*," *Process Biochem.* 39, 1561-1566.
- Reid, I. D. (1989). "Solid-state fermentations for biological delignification: Review," *Enzyme Microbiol. Technol.* 11, 786-803.
- Sanghi, A., Garg, N., Sharma, J., Kuhar, K., Kuhad, R. C., and Gupta, V. K. (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.
- Smith, D. C., and Wood, T. M. (1991). "Xylanase production by *Aspergillus awamori*, development of a medium and optimization of extracellular xylanase and β -xylosidases while maintaining low protease production," *Biotechnol. Bioeng.* 38, 883-890.
- Singh, S., Madlala, A. M., and Prior, B. A. (2003). "*Thermomyces lanuginosus*: Properties of strains and their hemicellulases," *FEMS Microbiol. Rev.* 27, 3-16.

- Singh, S., Tyagi, C. H., Dutt, D., and Upadhyaya, J. S. (2009). "Production of high level of cellulase-poor xylanases by wild strains of white-rot fungus *Coprinellus disseminatus* in solid-state fermentation," *New Biotechnol.* 26(3-4), 165-170.
- Singh, S., Dutt, D., and Tyagi, C. H. (2011). "Complete characterization of wheat straw (*Triticum aestivum* PBW-343 L. EMEND. FIORI & PAOL.) – A renewable source of fibers for pulp and paper making," *BioResources* 6(1), 154-177.
- Singh, J., and Garg, A. P. (1995). "Production of cellulases by *Gliocladium virens* on *Eichhorinia* under solid- state fermentation condition," *J. Indian. Bot. Soc.* 74, 305-309.

Article submitted: December 27, 2010; Peer review completed: February 25, 2011;
Revised version received and accepted: March 7, 2011; Published: March 8, 2011.