ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF CRUDE XYLANASE FROM *COPRINUS CINEREUS* AT-1 MTCC 9695 AND ITS EFFECTIVENESS IN BIODEINKING OF SOP

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Out of 10 fungal strains isolated from various sources, *Coprinus cinereus* AT-1 MTCC 9695 produced the highest xylanase (698.75 IU/mL), cellulase (1.01 IU/mL) and laccase (25.6±3.2 IU/mL) activities, using wheat bran and yeast extract as the carbon and nitrogen sources at incubation period of 7days, incubation temperature of 37 °C, pH 6.4 and solid substrate to NSS ratio of 1:3. The SSF was more effective, yielding 54.55% higher xylanase activity, compared to SmF. Biochemical characterization revealed that crude xylanase was active in the pH range of 6.0 to 8.0 with the maximum xylanase activity at pH 6.4 (699.60 IU/mL). At neutral and 7.5 pH levels, xylanase retained about 64 and 56% xylanase activity, respectively. At a temperature of 65 °C, xylanase retained about 74% of its optimum xylanase activity and at higher temperatures (75 and 85 °C), it retained 17 and 11% of its optimum activity, respectively. SDS–PAGE and zymogram analysis indicated that the molecular weights of xylanase produced by *Coprinus cinereus* AT-1 MTCC 9695 were of 50 kDa and 83 kDa. The effectiveness of crude xylanase was assessed by adding it along with cellulase in equal amounts (6 IU/mL) during deinking of SOP. It mitigated ERIC values and dirt counts by 11.34 and 2.20% and improved brightness, D_B and D_E by 3.07, 8.73 and 4.90%, respectively, compared to deinking with cellulase only.

Keywords: *Coprinus cinereus*, xylanase, cellulase, deinking, brightness, dirt counts

INTRODUCTION

Xylanase is a hydrolase that facilitates the complete hydrolysis of xylan. Xylan is a heteropolymer of β-1,4 linked D-xylopyranose residues and side chains, consisting of L-arabinofuranose, acetyl and glucuronic acid xylan. The xylan-degrading enzyme is a mixture of endo-β-1,4-xylanases (β-1,4-D-xylanxylanohydrolase; E.C. 3.2.1.8) and β-1,4-xylosidases (β-1,4-D-xyloside xylohydrolase; E.C. 3.2.1.37); along with α-glucuronosidase, acetylxylan esterase and phenolic acid esterase.[1] The most potent xylanase producers are the fungi,[2] especially, wood-rot fungi, since, they excrete enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria.[3] Among wood-rot fungi, white rotters are the most important as they can degrade all the wood cell wall components, including a highly recalcitrant polymer, lignin.[4] Though white-rot fungi have effective hemicellulases systems, yet, only a few studies have been made on their hemicellulases. Since biotechnological applications require large amounts of low-cost enzymes, an appropriate approach for xylanase production is the search for powerful xylanase producers and the use of cheap lignocellulosic substrates instead of expensive xylan.[5] In fact, lignocellulosics appear to be better substrates than xylan for xylanase production.[6]

It is well known that microbial systems manage their chemistry more efficiently than man-made chemical plants. Thus, the selected enzymes are more advantageous than chemicals for the desired modification of the pulp and very

prominent in the papermaking process. These enzymes include cellulases for enzymatic deinking,[7] xylanases for deinking, bleaching and enhanced drainage rates,[8] laccases and ligninases for delignification of pulp,[9] lipases, esterases for pith removal in the pulp and deinking of waste papers[10] and pectinases for various fiber handling operations and enzymatic retting of flax fibers.[11] The choice of the enzymes to be used was made depending on the type of the ink to be removed, as well as the type of the waste paper. The waste paper contains cellulose, hemicelluloses, starch added as dry strength additives and surface sizing agents, urea formaldehyde and melamine formaldehyde resins added as wet strength additives. Therefore, a concoction of enzymes having microbial cellulases,[7] hemicellulases,[7] amylases[12] and lipases[10] has shown promising results in deinking.

The pH of the writing and printing papers varies from 5.0 to 7.5 and depends upon the types of internal sizing (alum-rosin or alkyl ketene dimer and alkyl succinic anhydride). Therefore, xylanases working in acidic pH range are more effective for deinking. With this objective, the present study aims at investigating the potential of a strain of *Coprinus cinereus* to produce xylanase under SSF using the lignocellulosic substrates. The study includes the optimization of various physico-chemical parameters and the bio-chemical characterization of *Coprinus cinereus*. Finally, a comparison is made between cellulase and cellulase+xylanase deinked pulp of sorted office paper, in terms of brightness, dirt count and ERIC values, effluent characteristics and strength properties.

EXPERIMENTAL

Microbial strain and its growth conditions

Different fungal strains were isolated from lignocellulosic wastes, decomposing manure, sugarcane dumping sites, fruiting body and paper industry wastes. Samples were processed on wheat bran (3%) agar (2%) medium. The plates were observed for the appearance of fungal growth and fruiting bodies (indication of growth of basidiomycetes) were isolated from the plates. Fungal cultures were separately purified by subculturing on the basis of morphological features. Purified cultures were transferred on PDA slants at 37 °C for 5 days and stored at 4 °C. The cultures were maintained as a suspension of spores and fragments of fungal hyphae in 15% (*v*/*v*) sterile glycerol at -20 °C for long-term preservation.

Screening

Strains were primarily screened for xylanase production by viewing the zone of clearance on xylan agar medium (xylan – 1%, agar – 2%, dissolved in double-distilled water and autoclaved at 15 psi for 15 min). The crude enzyme extract (50 µL) obtained under solid-state fermentation conditions and each fungal isolate was placed separately into a 2-3 mm cut well in a solidified medium and incubated at 30 °C for 48 h. Then the plates were stained by Congo red dye and destained with 1M NaCl. Xylanase-producing strains were selected by observing the zone of clearance around the well against the red background. The aim of the primary screening was to select the fungal strain exhibiting the highest xylanase activity. Secondary screening was carried out on the basis of the highest xylanase activity with minimal cellulase activity in crude enzyme.

SEM

Detailed morphological studies of a fungal strain were carried out using SEM. The fungal mat was fixed using 3% (*v*/*v*) glutaraldehyde, 2% formaldehyde (4:1) for 24 h. Following primary fixation, the sample was washed thrice with double-distilled water. Then the samples were treated with alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. The samples were examined under SEM using the gold shadowing technique. Electron photomicrographs were taken at 15 kV, using SE1detector at desired magnifications.

SmF and SSF processes

Smf was carried out in 40 mL of NSS with 2% wheat bran in Erlenmeyer flasks (250 mL). The flasks were inoculated with cultures and incubated at 37 °C for 8 days in incubator shaker with constant shaking (120 rpm). Alike, SSF was performed as described by Beg *et al.*[13] A slurry of fermentation medium containing 5 g of wheat bran and 15 mL of NSS was added in a solid to liquid ratio of 1:3 in Erlenmeyer flasks (250 mL) and incubated with fungal cultures. The cultures were incubated at 37 °C for 8 days. In the SmF process, the enzyme was directly filtered through a four-layered cheese cloth, while in the SSF, the enzyme was extracted using 15 mL of distilled water. The contents of the flask were crushed and shaken for 30 min to harvest the enzyme from the fungal cells. The whole contents were then filtered through a four-layered cheese cloth. The filtrate obtained was centrifuged (Sigma centrifuge model 2K15) at 5000 g for 10 min and a temperature of 4 °C. The supernatant was used as crude enzyme and was stored at –20 °C until used. The enzyme was harvested and assayed for xylanase[14] and cellulase activities[15] as per standard protocol. Protein concentration was estimated according to Lowery *et al.*,[16] with BSA as a standard. Laccase assay was performed using ABTS as substrate.[17]

Optimization of incubation period, temperature and pH

A set of Erlenmeyer flasks (250 mL), containing 5 g of wheat bran and 15 mL of NSS (pH 6.0), were autoclaved at 15 psi for 15 min and inoculated aseptically with fungal culture. Then, they were incubated at 37 °C and harvested after 2nd to 11th day of incubation with an interval of one day.

Similarly, the effect of temperature on the production of xylanase was studied by incubating the inoculated flasks at different temperatures varying from 27 to 52 °C with an interval of 5 °C for 7 days. Likewise, in order to study the effect of pH on xylanase production, the pH of NSS solution was adjusted between 4.6 to 11.2 with an interval of 0.6 by 1 N $NaOH/H_2SO_4$. The contents of the flasks were autoclaved at 15 psi for 15 min, inoculated aseptically and incubated at 37 °C, and then harvested after 7 days of incubation.

All these enzyme preparations were assayed for protein concentration,[16] xylanase[14] and cellulase activities[15] as per standard protocols.

Effect of carbon and nitrogen sources

Different agricultural residues, like wheat bran, sugarcane bagasse, wheat straw and rice straw were powdered in a laboratory Wiley mill and the fractions retained on a +100 mesh were used as substrates for xylanase production. Five grams of each finely powered agricultural residue was taken in an Erlenmeyer flask of 250 mL capacity. Similarly, one g of five different nitrogen sources, including yeast extract, malt extract, peptone, soya bean meal and beef extract, was added separately in one Litre of NSS and used for xylanase production. The pH of all the flasks containing NSS was adjusted to 6.4 and fermentation was carried out for 7 days at 37 °C. The crude enzymes were assayed for xylanase[14] and cellulase activities[15] per standard protocols.

Effect of glucose and lactose concentration and optimization of moisture level

The incubation medium was supplemented with 1-5 g/L of glucose/lactose in NSS solution, and wheat bran to NSS ratio was maintained at 1:3. The flasks were inoculated and incubated at optimum incubation period, temperature and pH and the effect of glucose/lactose was studied on xylanase titre. The influence of moisture level on the xylanase titre was evaluated by varying the ratio of wheat bran to NSS (1:2, 1:2.5, 1:3, 1:3.5 and 1:4.0 in *w*/*v*). The fermentation was carried out for 7 days at 37 °C. The crude enzymes were assayed for xylanase[14] and cellulase activities[15] per standard protocols.

Optimum pH and pH stability

The pH stability was determined in the pH range of 6.0-9.0 by incubating the enzyme in buffers of different pH (potassium-phosphate, pH range: 6.0-7.4; and borax-boric acid, pH range: 7.6-9.0). After 15 min of incubation, the residual xylanase activity[14] of the crude enzyme samples was determined under standard assay conditions.

Optimum temperature and thermostability

The thermostability of the enzyme was determined by incubating the crude enzyme preparations at temperatures ranging between 55-85 °C for up to 15 min. The samples were withdrawn after 15 min and analyzed for residual xylanase activity[14] under standard assay conditions.

SDS-PAGE and zymogram analysis

SDS-PAGE of the test sample was carried out using a concentrated crude culture filtrate as sample. Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was carried out for visualization of xylanase activity *in situ*. Xylan (substrate) at a concentration of 0.1% was incorporated into separating gel before adding the TEMED for polymerization. The enzyme samples were mixed with sample buffer lacking SDS and β-mercaptoethanol. After electrophoresis, the gel was soaked with 0.1% (*w*/*v*) Congo red for 5 min with mild shaking. The excess of dye was decanted and the gel was washed with 5% (*w*/*v*) NaCl until the excess of stain was totally removed and the background was clear.

Mass production of xylanase

Optimizing the fermentation conditions, mass production of xylanase was carried out by the SSF process in order to validate the effectiveness in biodeinking sorted office paper. Forty g of wheat bran was taken in a 2 L flask, moistened with 120 mL of NSS (pH 6.4) and the slurry was inoculated with 16 disks of 5 mm diameter from the actively growing culture of fungal strain AT-1. The flask was harvested after the 7th day of incubation at 37 °C. The crude enzyme extract obtained was used for the determination of protein concentration,[16] xylanase[14] and cellulase activities[15] per standard protocols.

Waste paper collection, characterization and pulping

SOP was collected from M/s Khatema Fiber Ltd., Khatema, District Udham Singh Nagar (U.K., India). SOP consists of white and colored ground-wood-free paper; unbleached fiber may include a small percentage of ground-wood computer printout and facsimile papers coated with toner and laser printing, and industrial papers. The total prohibitive materials and throw outs were of 3% in SOP.[18] Ash contents (TAPPI T 211 om-02 "Ash in wood, pulp, paper and paperboard: combustion at 525 °C") were of 20.72% in SOP. The moisture content (TAPPI T 208 wd-98 "Moisture in wood, pulp, paper and paperboard by toluene distillation") was of 10% in SOP. [19] SOP was torn into small pieces and soaked in warm water at 50

°C for 30 min. Pulping was done in a hydrapulper of 500 g capacity (Weverk, A-47054, Sweden), with motor speed and temperature control. SOP was pulped for a pulping time of 20 min, temperature of 60 °C and consistency of 12% in the presence of a surfactant (oleic acid) dose of 0.05% and at pH 7.2±2. The rotor speed of the hydrapulper was adjusted to 650 rpm.

Enzyme treatment and ink floatation

The pulp produced under optimum conditions was treated with different doses of cellulase from *Aspergillus niger* AT-3 (CMC_{ase}, FP_{ase} and β-glucosidase activities of 25.12, 2.23 and 0.87 IU/mL, respectively),[20] and xylanase from *Coprinus cinereus* AT-1 at a consistency of 12%, pH 5.3±2, temperature of 55±2 °C, reaction time of 60 min and surfactant (Tween 80) dose of 0.1%. Enzymatically treated pulp was washed with tap water on a Weverk laboratory flat stationary screen having 300 mesh wire bottom for the removal of hydrolysed chemicals and subjected to ink floatation at a floatation time of 10 min, consistency of 1%, temperature of 35±2 °C and pH 7.2±2. After ink floatation, the pulp was washed with warm water and a pulp pad was prepared for brightness determination (TAPPI T 218 sp-02 "Forming handsheets for reflectance testing of pulp [Büchner funnel procedure]" and was evaluated for brightness (TAPPI T452 om-02 "Brightness of pulp, paper and paperboard [Directional reflectance at 457 nm]") and ERIC (TAPPI T567 pm-97 "Determination of ERIC by infrared reflectance measurement"). After ink floatation, the pulps were defibered in a WEVERK disintegrator and evaluated for CSF (TAPPI T 227 om-99 "Freeness of pulp [Canadian standard method]"). Laboratory handsheets (60 g/m^2) were prepared on a British sheet former (TAPPI T 205 sp-02 "Forming handsheets for physical tests of pulp"), conditioned at a relative humidity of 65±2% and temperature of 27±1 ºC, and evaluated for burst index (TAPPI T 403 om-97 "Bursting strength of paper"), tensile index (TAPPI T 494 om-01 "Tensile properties of paper and paperboard [using constant rate of elongation apparatus]"), double fold (TAPPI T 423 cm-98 "Folding endurance of paper [Schopper type tester]"), tear index (TAPPI T 414 om-98 "Internal tearing resistance of paper [Elmendorf-type method]") and pulp viscosity (TAPPI T 230 om-04 "Viscosity of pulp [capillary viscometer method]") and compared with the control.[19] Similarly, laboratory-made handsheets were evaluated for dirt counts (TAPPI T 213 om-01 "Dirt in pulp") and deinkability factors, i.e. D_B (based on brightness) and D_E (based on ERIC), respectively, after ink floatation using the following equations:

$$D_B, \% = \frac{B_F - B_P}{B_B - B_P} \times 100 \quad (1)$$

where:

B_P = Brightness after pulping, % (ISO)

B_F = Brightness after floatation, % (ISO)

B_B = Brightness of the sample paper (blank), % (ISO)

D_B = Deinkability factor based on brightness, % (ISO)

$$D_E, \% = \frac{E_P - E_F}{E_P - E_B} \times 100 \quad (2)$$

where:

E_B = ERIC value in the absence of ink particles (blank)

E_F = ERIC value after floatation deinking

E_P = ERIC value of the sample sheet before ink removal (after pulping)

D_E = Deinkability factor based on ERIC value, %

Filtrates collected from the enzymatic treatment and ink floatation stages were mixed in equal amount and combined effluents were analyzed for COD (IS 3025: Part 58, 2006: COD-Closed reflux titrimetric method using Thermo reactor CR2010, BOD (IS 3025: Part 44: 2006 and colour)[21] and total solids (IS 3025: Part 15, 2003-Total residue (total solids – dissolved and suspended solids).[22]

Statistical analysis

All experiments were carried out in triplicate and the experimental results were represented as the mean ± standard deviation of three identical values.

RESULTS AND DISCUSSION

Morphological identification and morphological characteristics of *C. cinereus* AT-1

Table 1 reveals the morphological characteristics of 10 fungal strains collected from different lignocellulosic sources. In the present investigation, moist wheat bran is used in the medium as a sole carbon source, as it has already been shown to be a promising carbon source for xylanase production.[23] The formation of fruiting bodies and white thread, like mycelial network, on the decaying wood and aerial fruit bodies, after successive degradation indicates the growth of basidiomycetes (Plate 1A). When decaying wood was buried in moist wheat bran and incubated at 37 °C, it showed mycelial growth, spores and the appearance of a colony (Plate 1B). The xylanase production ability of the fungal strain on the xylan-agar (XA) plate was observed after primary screening. Seven out of ten isolates result in a clear zone formation on the xylan-agar plates, of which AT-1 result in a clear zone formation on the XA-plate when stained with Congo red (Table 1). These isolates were selected as xylanase producers. These seven strains were further subjected to secondary screening for their xylanase production ability in SSF condition (Table 1). Out of seven, fungal strain AT-1 produces the maximal xylanase activity, i.e. 467.00 IU/mL, with a minimum cellulase activity,

i.e. 0.80 IU/mL. Fungal isolate AT-1 was selected for further studies based on its higher xylanase activity.

Table 1
Morphological characteristics of different fungal isolates

Isolated strains	Sources	Mycelia color	Spore color	Clear zone diameter, mm	Xylanase activity, IU/mL	Cellulase activity, IU/mL
AT-01	Dead and decaying wood	White	Black	16.8	467.00±9.1	0.94±0.12
AT-13	Dead and decaying wood	White	Brown	12.2	360.80±8.7	0.80±0.11
AT-14	Decomposing manure	White	Black	10.5	166.50±8.2	1.49±0.14
AT-15	Dead and decaying wood	Green	Green	—	—	
AT-17	Paper industry waste	White	Brown	10.5	138.20±9.1	1.41±0.16
AT-18	Paper industry waste	Black	Black	—	—	
AT-19	Fruiting body	White	No spore formation	6.5	85.96±6.2	1.82±0.18
AT-20	Sugarcane dumping site	Yellowish white	No spore formation	—	—	
AT-21	Decomposing manure	Dirty white	Black	3.7	57.37±5.0	0.97±0.13
AT-22	Mango tree bark	Creamish white	No spore formation	1.4	23.55±3.5	1.03±0.15

± refers to standard deviation

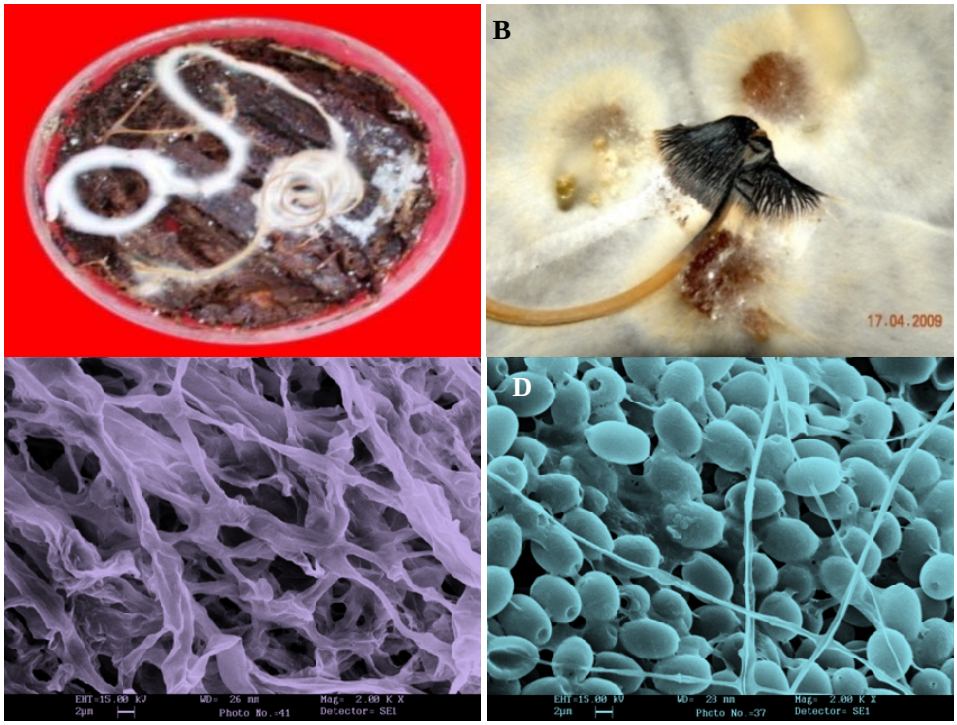

Plate 1: (A) Formation of fruiting bodies, (B) Formation of basidiospore, (C) SEM of hyphae of *Coprinus cinereus* AT-1 at a magnification of 2.00 KX (D) SEM of club-shaped basidiospores of *Coprinus cinereus* AT-1 at a magnification of 2.00 KX

The selected fungal isolate AT-1 was morphologically identified as a wild mushroom from Indian Type Culture Collection (ITCC), Plant Pathology Division, IARI, New Delhi (India). The mycelia of the fungal strain AT-1, which was identified as *Coprinus cinereus* AT-1, are white in colour, while the basidiopores produced are blackish in colour. The fungal strain *C. cinereus* AT-1 shows a smooth colony without any distinct growth rings. The fruiting bodies indicate that the fungi are related to the basidiomycetes family, which ultimately lead to spore formation on the 11th day of incubation. The finer structural details of the mycelia and spores as observed through SEM are shown in Plate 1C. The hyphae of *C. cinereus* AT-1 are thin, elongated and straight. The presence of basidium, basidiospores, pileocystidea and hymenial layers are shown in Plate 1D. The basidiospores produced by *C. cinereus* AT-1 are club-shaped, which is a characteristic feature of all the members of the basidiomycotina group. In a natural habitat, a cap is present in wild mushrooms, initially white in colour, but soon beginning to turn to grayish brown, with a brownish center (Plate 1D). It is easily recognized as a wild mushroom by its cap shape and grayish black gills. It is an extremely fragile mushroom, and the caps quickly crumble when handled.

Molecular identification

For the identification of fungal isolate wild mushroom AT-1 up to a species level, molecular identification was done at MTCC (IMTECH, Chandigarh). The NCBI-BLAST search analysis of the DNA sequence-data indicated that the fungus is a member of *Coprinus cinereus.* The fungal isolate was deposited in MTCC with an accession number 9695. The DNA sequence data of the fungal isolate *Coprinus cinereus* AT-1 is shown as under:

Coprinus cinereus isolate AT-1, 28S large subunit ribosomal RNA gene, partial sequence *Coprinus cinereus* isolate AT-1, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence,

GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAAT
CTGGCAGTCTTTGGCTGTCCGAGTTGTAAACTAGAGAAGTGTTACCCGCG
TCGGACCGTGTACAAGTCTCCTGGAATGGAGCATCATAGAGGGTGAGAAT
CCCGTCTTTGACATGGACTACCGAGGCTTTTGTGGTGCGCTCTCAACGAG
TCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAAATTCCATCTAAA
GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGA
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TACATCGGTTGGGACTGAGGAATTCAGCACGCCGCAAGGCCGGGTTTCGA
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CCCGGACGCGTAATGAAAGTGAAAGTCAGGATCCCTGTCATGGGGAGCAC
TGGCGCCCGCACCTGAAGTTTACGGACGGATGTGAGGTAAAGCATGTATG
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AAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGCAAATCGATCGTCGA
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GATATGCTTAAGTTCAGCGGGTAGTCTTGCCTGATTTGAGGTCAAAATTG
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ACTAATACATTTCAGAGGAGCAGACCACGAGAGTGGACCTGCAACCCCCA
CATCCAAGCCTGCACACAACAAAGTTGGTGAGGTTGAGAATTTAATGACA
CTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAA
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GCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTG
TATAGGTTTATAGGCACAAGGCCTTGAGATGACATTCCATAACATTCAGT
TTGGGGTGTGTAAGAAATCATAGACCTGGAAATTCATGGCAAGCCGGCCT
TCTTTCGAAAGCGACAGCAAGCCACGCATCCGCCTTGCGACGAGAGGTAT
CCAGGCCTACAGTGTGTGCACAGGTGGAGAAATAAAGGTGACGGGCGTGC
ACATGCTCCCGGGGGAGCCAGCGACAACCACGTCAGATTTATTCAATA

Comparative analysis of different fermentation conditions

The xylanase and cellulase activities of fungal strain AT-1 are of 475.50 and 0.84 IU/mL, respectively, under SSF. On the other hand, the cellulase activities of fungal strain AT-1 are of 224.10 and 0.48 IU/mL, respectively, under SmF. The xylanase and cellulase activities of strain *C. cinereus* AT-1 under SSF conditions are 54.55 and 42.86%, respectively, higher than those under SmF conditions (Figure 1). Agnihotri *et al.* achieved similar results with *Coprinellus disseminatus* SW-1 NTCC 1165 using wheat bran under SSF.[23] The aim of SSF is to bring the cultivated fungi into a tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation. Therefore, the increased yield of xylanase produced by the fungal strain *C. cinereus* AT-1 observed in SSF may be attributed to close contact between mycelium and substrate, which is not possible during SmF. That is why, SSF has been chosen for further optimization studies for crude xylanase production by the fungal strain *Coprinus cinereus* AT-1.

Optimization of incubation period, temperature and pH

The enzyme production increases with increasing incubation time and attains its maximum level on the 7th day of incubation, i.e. 615.05 IU/mL. Further, on increasing the incubation period beyond 7 days, the xylanase activity of *C. cinereus* AT-1 decreases, while protein biomass continues to increase from the 1st to the 7th day and then becomes nearly constant with a small decrease after the 10th day (Figure 2A). Likewise, cellulase production also increases to its maximum level after 7 days of incubation, i.e. 0.92 IU/mL.

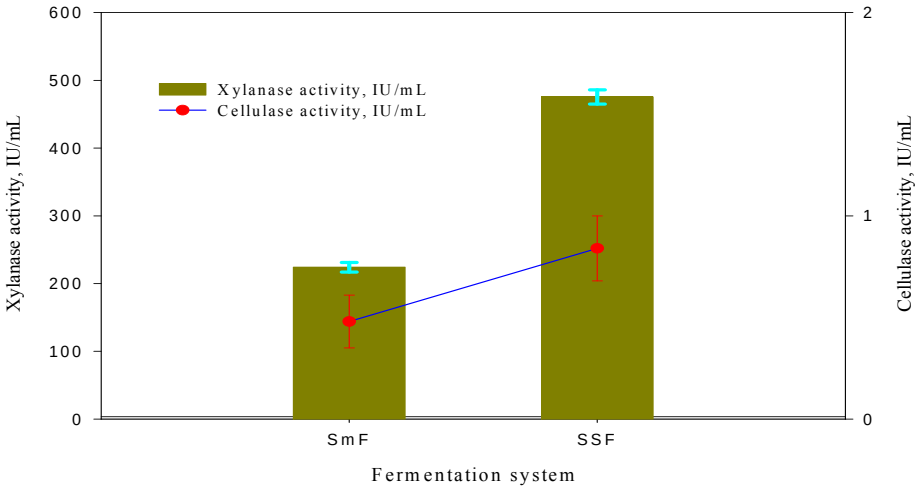
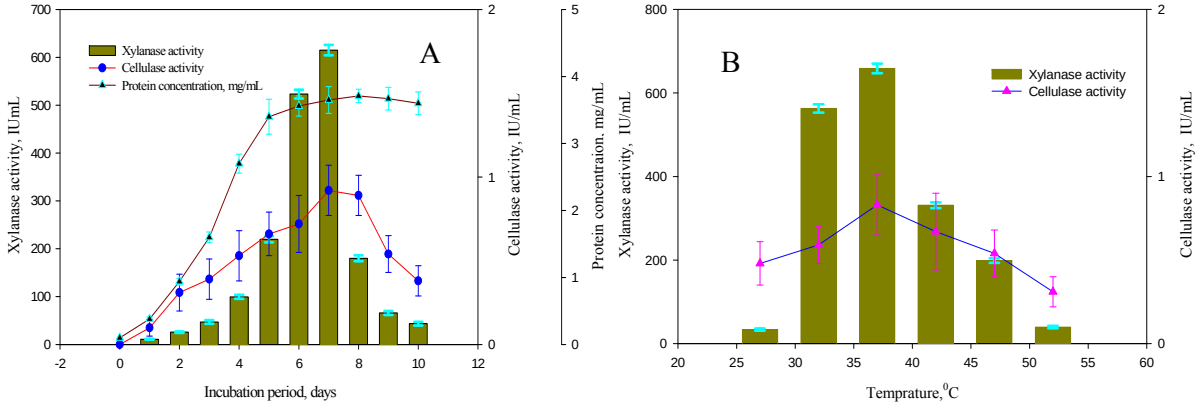


Figure 1: Comparison of fermentation systems for enzyme production by *C. cinereus* AT-1



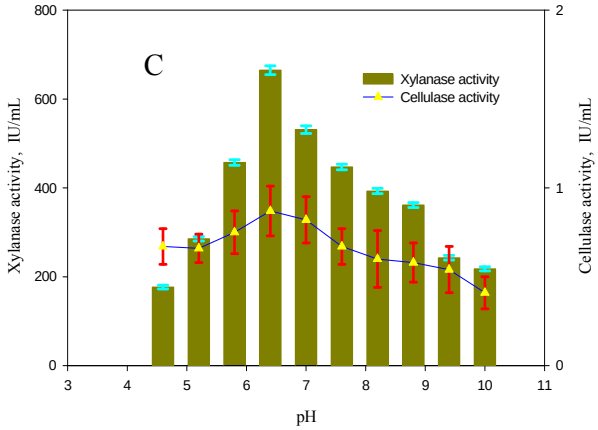


Figure 2: Optimization of (A) incubation period (B) temperature and (C) pH for enzyme production by *Coprinus cinereus* AT-1

On longer incubation, the enzyme activity of the fungal strain decreases, while protein biomass continues to increase from day the 1st to the 7th and then becomes nearly constant with a small decrease after the 10th day. It means that the enzyme production is dependent on biomass, but only during the exponential phase of fungal growth.

Since cellulase and xylanase are a part of primary metabolites, they are produced during the exponential phase of growth, and at the onset of the death phase, the enzyme secretion starts to decrease. Xylanase production is maximal at the end of the exponential phase (7th day) and then slowly starts decreasing at the onset of the death phase. Basidiomycetes are slow-growing fungi, so xylanase production is maximum after 7 days in *Pleurotus ostreatus*,[3] *Coprinellus disseminatus* SW-1 NTCC 1165,[23] and *Trichoderma harzianum* 1073 D3,[24] after 6 days in *Phanerochaete chrysosporium*[25] and after 8 days in *Volvariella diplasia*.[26]

The maximum xylanase activity, i.e. 658.57 IU/mL, in the case of *C. cinereus* AT-1 is found at 37 °C , while the lowest, i.e. 33.60 IU/mL, at 27 °C (Figure 2B). While moving towards the extreme ends of the optima, the activities decrease since the fungal growth ceases. The maximum xylanase activity at 37 °C for the fungal strain *C. cinereus* AT-1 suggests that the test isolate is mesophilic in nature. The cellulase activity of the fungal strain also increases with increasing the incubation temperature up to 37 °C and then declines. The decrease in the xylanase production level is possibly due to the lower transport of substrate across the cells at a lower temperature, which may be the reason for the lower yield of the product. At a higher temperature, the energy requirement of cellular growth is high due to the thermal denaturation of the enzymes of the metabolic pathway resulting in lower production of the metabolites.[27] A thermostable and cellulase-free xylanase is produced by *Streptomyces* sp. QG-11-3 in the SSF using wheat bran as carbon source at 37 °C.[13] Xylanase production is found optimum at 37 °C under SSF for *Bacillus pumilus* ASH using wheat bran as substrate,[28] while the highest xylanase activity by *B. licheniformis* A99 is observed at 50 °C under SSF using wheat bran as carbon source.[29]

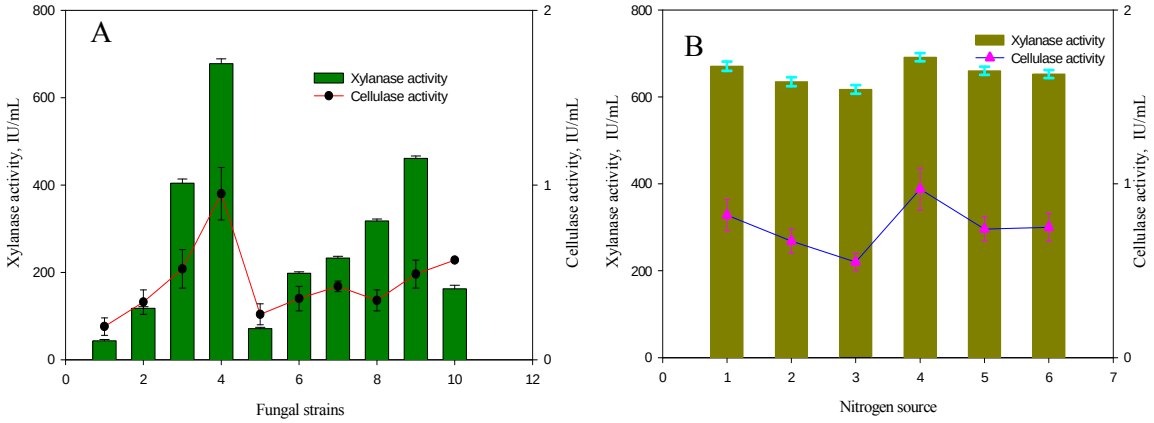
The xylanase activity of the fungal strain *C. cinereus* AT-1 increases with increasing pH from 4.6 to 6.4 while keeping other fermentation conditions constant, however, when further increasing pH the xylanase activity decreases steadily. Three distinct phases as a function of pH are illustrated in Figure 2C: at low pH (4.6 to 5.8), there is an increase in enzyme activity, at high pH (above 7.6) there is a decrease and at an intermediate (optimal) pH range (6.4-7.0), the activity is maximum, thereby leading to a characteristic bell-shaped curve. The maximum cellulase activity of strain AT-1 (0.87 IU/mL) is observed at pH 6.4, while the minimum at pH 10 (0.41 IU/mL). The H^+ concentration in the fermentation medium has a profound effect on the xylanase production. Microorganisms may need to adapt their function in order to cope with a change in H^+ concentration. If this change is too abrupt, the response of microbes may lag behind or overshoot. Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium. Thus, a modification in the pH may also cause

some micronutrients to precipitate and become impossible to assimilate.[30] Fermentation at lower and higher pH proves to be detrimental perhaps because of the inactivation of the enzyme system. Since, enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are likely to be affected by pH changes and the catalytic property of the enzyme is markedly influenced. The reason for poor production at higher pH is probably the proteolytic inactivation of the xylanase. The optimum pH for the xylanase production[31] by *A. versicolor* is 6.5 under SmF at 30 °C.

Effect of carbon and nitrogen sources and glucose and lactose concentrations

The effect of different lignocellulosic substrates (cheap agricultural wastes, including sugarcane bagasse, wheat straw, rice straw and wheat bran) as the sole carbon source on xylanase production by *C. cinereus* AT-1 is graphically represented in Figure 3A. The highest xylanase activity (677.85 IU/mL) is found when wheat bran is used as the sole carbon source. The enzyme activity in other carbon sources decreases in a descending order: rice straw (404.03 IU/mL), wheat straw (117.86 IU/mL) and sugarcane bagasse (43.36 IU/mL), respectively. The addition of wheat bran to rice straw in the ratio of 1:1 improves xylanase production (461.00 IU/mL) by *C. cinereus* AT-1 to a great extent, in other mixtures of carbon sources, xylanase production reducing in the following descending order: bagasse+wheat bran>wheat straw+wheat bran>wheat straw+rice straw>bagasse+rice straw>bagasse+wheat straw. In the same way, the cellulase activity of the fungal strain is the highest with wheat bran (0.95 IU/mL) and decreases in the following descending order: rice straw>wheat straw>bagasse, while in mixtures it decreases in the following descending order: rice straw +wheat bran>wheat straw+wheat bran>sugarcane bagasse+wheat bran>sugarcane bagasse+rice straw>wheat straw+ rice straw>sugar cane bagasse+wheat straw. Hence, based on the results, wheat bran has been found to be the best substrate for xylanase induction, because it contains sufficient nutrients and remains loose in moist conditions, thereby providing good aeration and large surface area,[29] which can be used by the microbes for their growth and metabolic activity.[32] Wheat bran has been described as a potent substrate for the xylanase production under SSF by *Streptomyces cyaneus* SN32,[33] *Bacillus pumilus*,[34] *Bacillus subtilis*,[35] *Bacillus pumilus* ASH[28] and *Coprinellus disseminatus* NTCC 1165.[23]

Figure 2B shows that all the tested nitrogen sources (urea, yeast extract, peptone, malt extract, soya bean meal and beef extract) stimulate the production of xylanase from *C. cinereus* AT-1. Yeast extract is the best nitrogen source for both xylanase (691.40 IU/mL) and cellulase (0.97 IU/mL) production. In the presence of more available nitrogen, the mycelium grows better and its activity also increases.[36] Singh *et al*. obtained the maximum xylanase production for the fungal strain *Coprinellus disseminatus* SH-1 NTCC-1163 and SH-2 NTCC-1164[37] with yeast extract as nitrogen source, while Agnihotri *et al*. reported soya bean meal to be the most favorable nitrogen source for the basidiomycetes strain *Coprinellus disseminatus* SW-1 NTCC-1165.[23]



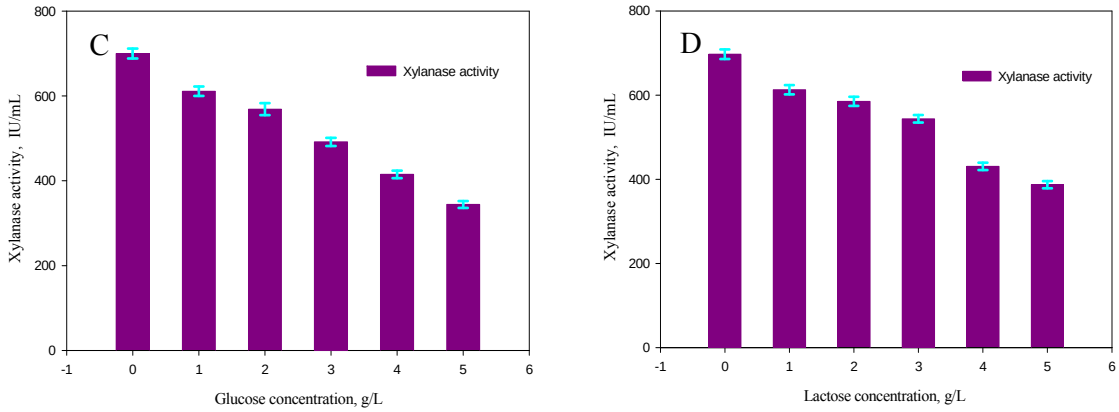


Figure 3: Effect of (A) carbon sources (1–Bagasse, 2–Wheat straw, 3–Rice bran, 4–Wheat bran, 5–Sugarcane bagasse+wheat straw, 6–Sugarcane bagasse+rice straw, 7–Sugarcane bagasse+wheat bran, 8–Wheat straw+rice straw, 9–Wheat straw+wheat bran, 10–Rice straw+wheat bran); (B) nitrogen sources (1–Urea, 2–Peptone, 3–Malt extract, 4–Yeast extract, 5–Beef extract, 6–Soya bean meal (C) glucose concentration and (D) lactose concentration during crude xylanase production by *Coprinus cinereus* AT-1

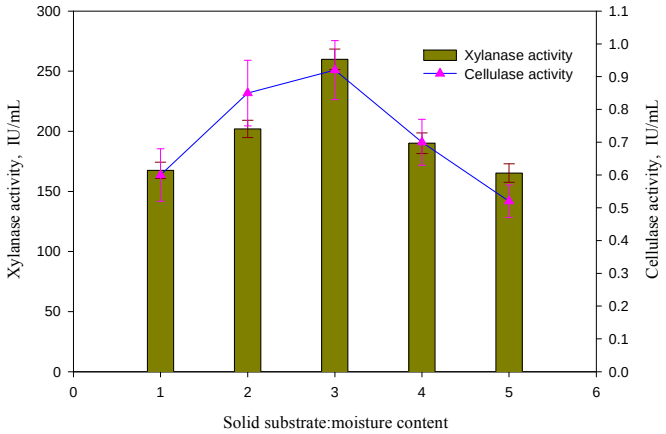


Figure 4: Optimization of solid substrate:moisture content for cellulase production by *Coprinus cinereus* AT-1 (1–1:2.0; 2–1:2.5; 3–1:3.0, 4–1:4.0)

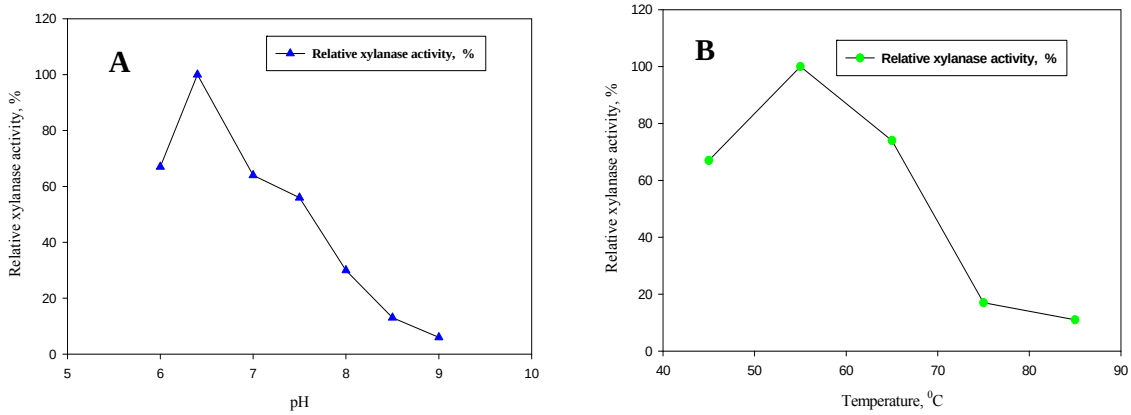


Figure 5: Stability of (A) pH (B) temperature of crude xylanase produced by *Coprinus cinereus* AT-1

Figures 3C and D reveal that xylanase production by *C. cinereus* AT-1 decreases with increasing glucose and lactose concentration from 1 to 5 g/L. The addition of glucose and lactose

represses xylanase production, which may be possibly due to catabolite repression.[38] Catabolite repression of xylanase production by glucose was also observed by Sanghi *et al*.[35] in *Bacillus subtilis* ASH.

Optimization of moisture content for xylanase production

Figure 4 shows that the maximum xylanase titre (697.90 IU/mL) is obtained when wheat bran is moistened with NSS in a ratio of 1:3, which declines with a further increase in the substrate to moisture ratio. Similarly, the maximum cellulase activity (0.92 IU/mL) has been found at a solid substrate to moisture content ratio of 1:3. In SSF, when using wheat bran and eucalyptus Kraft pulp as primary solid substrates, *Streptomyces sp*. QG-11-3[13] produced the maximum xylanase yield at a substrate to moisture ratio of 1:2.5 and 1:3, respectively. In contrast, a lower solid substrate to moisture level of 1:1 has been reported for the maximum xylanase production by *Bacillus* sp. A-009.[39]

Effect of pH on the activity and stability of xylanase

Figure 5A shows the effect of pH on the xylanase activity of *C. cinereus* AT-1 at a temperature of 55 °C for 15 min of reaction time. It shows that the crude xylanase produced by *C. cinereus* AT-1 is active in the pH range of 6.0 to 9.0 with the maximum xylanase activity at pH 6.4 (699.60 IU/mL). At pH 6.0, 67% of the maximal activity is maintained. Above pH 6.4, a decrease in xylanase activity is recorded. At a neutral pH (7.0), xylanase retains about 64% of its optimum activity, while at a pH of 7.5, 56% of the optimum activity is retained. Even at pH 8.0, the enzyme can retain 30% of its optimum activity, which shows its alkali-tolerant nature. Enzymes are proteins in nature. Under harsh conditions, such as change in pH, high temperature or in the presence of high concentrations of metal ions, proteins tend to lose their basic structure (denaturation), subsequently, losing active sites, which in turn results in a loss of enzyme activity. Besides this, the pH activity profiles of enzymes is highly dependent on the pKa value of the catalytic residues, which depends on the local environment and hence on the nature of the amino-acids in the vicinity of the catalytic residues. Studies carried out with other fungal species[1] also concluded that the most suitable pH value for xylanase activity is within the acidic region, which supports our findings.

Effect of temperature on the activity and stability of xylanase

Figure 5B shows that the optimum temperature for the crude xylanase activity of the fungal strain is 55 °C (702.00 IU/mL) and beyond it xylanase activity decreases. At the temperature of 65 °C, xylanase from *C. cinereus* AT-1 retains about 74% of its optimum xylanase activity. When assayed at higher temperatures, i.e. 75 and 85 °C, the xylanase loses most of its activity, retaining just 17 and 11% of its optimum activity, respectively. The experimental results indicate the thermo-tolerant nature of xylanase produced by *C. cinereus* AT-1. The thermostability of enzymes seems to be a property acquired by a protein through the combination of many small structural modifications that are achieved with the exchange of some amino-acids. The variation of the canonical forces, e.g. hydrogen bonds, ion-pair interactions and hydrophobic interactions, provide thermozymes with resistance to high temperature.[40] The crude xylanase produced by *Aspergillus oryzae* has the temperature optimum between 60 and 65 °C. After 10 and 30 min of incubation at 60 °C, the residual activity is 40 and 10%, respectively, of the optimum activity for *Aspergillus oryzae*.[41]

Mass production of crude xylanase

The mass production of crude xylanases was done in 1000 mL flasks under fermentation conditions, optimized as described above and the results are summarized in Table 2. *Coprinus cinereus* AT-1 shows xylanase, cellulase and laccase activities of 742.47, 0.98 and 25.9 IU/mL, respectively, and a protein concentration of 5.8 mg/mL.

Molecular characterization by SDS-PAGE analysis

Plates 2A and B reveal that two bands showing xylanolytic activity were detected as the hydrolysis zones in the zymogram during the electrophoresis of the enzyme. The molecular weights of these proteins were found to be about 50 kDa and 83 kDa, respectively, for fungal strain *C. cinereus* AT-1. The xylanase isolated from strain *C. cinereus* AT-1 belongs to the high molecular weight category.

Table 2
Mass production of extracellular enzymes by *Coprinus cinereus* AT-1 under optimized conditions

Fungal strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
Coprinus cinereus AT-1	698.75±11.7	1.01±0.15	25.6±3.2	5.7±1.1

± refers to standard deviation

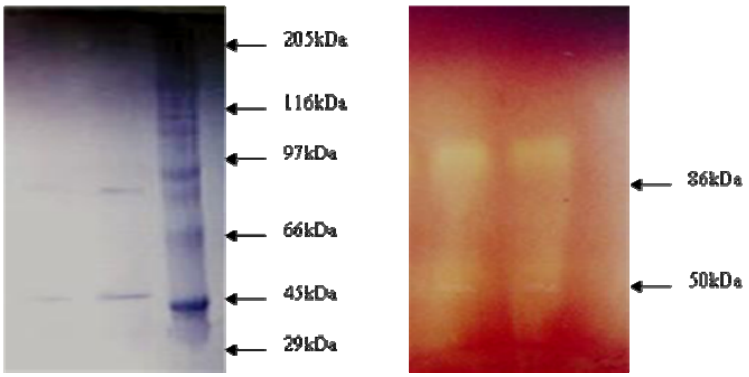


Plate 2: SDS-PAGE (A) and zymogram (B) of xylanase produced by *Coprinus cinereus* AT-1

Effectiveness of xylanase in biodeinking

When treated with crude cellulase before ink floatation, SOP pulp mitigates the ERIC value and dirt counts by 170.84 and 82.3%, respectively, whereas it improves brightness, D_B and D_E by 8.13, 23.10 and 76.30%, respectively, compared to the control (Table 3). Woodward *et al.*[42] explains that cellulase binding on pulp fiber may result in a surface fiber alteration, which is sufficient to favour ink detachment during repulping. Nevertheless, other authors report that the main effect is the hydrolysis and superficial degradation of cellulose, which implies ink removal from fibers.[43] The introduction of xylanase in cellulase-assisted deinking improves deinking efficiency as it mitigates the ERIC value and dirt counts by 11.34 and 2.20%, whereas it improves brightness, D_B and D_E increase by 3.07, 8.73 and 4.90%, respectively, compared to cellulase treatment followed by deinking. The removal of non-fibrous additives, like wet strength resins, fillers and sizing agents like alum-rosin etc., during stock preparation of SOP improves pulp freeness (+8.90%) and pulp viscosity (6.19%) of cellulase-deinked pulp, compared to the control. Further, the addition of xylanase during cellulase deinking of SOP improves pulp freeness and pulp viscosity by 0.80 and 3.51%, respectively, compared to cellulase-deinked pulp. The increase in viscosity is due to the high specific surface area of the tertiary fines (generated during waste paper pulping) and the attack of cellulase was very specific towards tertiary fines. The combined effluent generated during cellulase deinking of SOP shows an increase in total solids (11.25%), BOD (68.82%) and COD (64.10%) (Table 3). It may be due to the hydrolysis of fibrils attached with ink particles and to the removal of additives/contaminants added during stock preparation of SOP. Magnin *et al.*[44] has also reported an increase in COD as a result of enzymatic treatments, which may be due to the hydrolytic property of the enzymes. The enzyme releases soluble sugars from the pulp to the process water, thus increasing COD. Total solids, BOD and COD are further improved in cellulase+xylanase deinking of SOP pulp due to the hydrolysis of xylan and cellulose fibers, which are attached with ink particles, compared to cellulase-based deinking. The improvement in the strength properties of cellulase-deinked SOP may be due to the peeling effect.[45] Cellulase also plays an important role in reducing the refining energy, as well as improving the mechanical strength properties of enzymatically treated pulps. Pulp fibrillation by cellulase was recognized as a means to enhance strength properties by Bolaski and his co-workers.[46] The removal of xylan during cellulase+xylanase-based deinking of SOP mitigates the tensile and burst indexes more, compared to cellulase-based deinking of SOP, but there is an improvement in tear index. Enzyme treatment hydrolyzes xylan (low molecular weight) from the pulp and results in an increase in

the average molecular weight of the polymer system. Therefore, tear index slightly improves, whereas other properties, like burst and tensile indexes, depending upon hydrogen bonding, decrease due to depolymerization of xylan.[47]

Table 3
Effect of cellulase and cellulase+xylanase on effectiveness of biodeinking

Characteristics	Results after pulping			
Brightness, %	61.80±0.85			
ERIC, ppm	283.20±6.9			
Enzyme treatment stage	Control	% change in properties compared to control, + or –		% change in properties due to addition of xylanase, + or –
		Cellulase, 6 IU/mL	Cellulase + xylanase, 6+6 IU/mL	
Results after ink floatation***				
Total pulp yield, %	82.80±1.4	–2.70	–3.80	–1.10
Brightness, %	64.80±0.90	+8.13	+11.20	+3.07
Deinkability (D_B), %	8.52±0.15	+23.10	+31.83	+8.73
ERIC, ppm	276.23±8.74	–170.84	–182.18	–11.34
Deinkability (D_E), %	5.0±0.10	+76.30	+81.20	+4.90
Dirt count, mm^2/m^2	20181±65	–82.30	–84.5	–2.20
CSF, mL	510±2.0	+8.90	+9.73	+0.80
Pulp viscosity, cm^3/g	422.30±5.9	+6.19	+15.65	+3.51
Characteristics of effluent				
Total solid, mg/L	1.42	+11.25	+13.94	+2.69
COD, kg/tonne	23.4	+64.10	+64.6	+0.50
BOD, kg/tonne	8.0	+68.82	+69.58	+0.76
Strength properties				
Tensile index, Nm/g	22.44±1.1	+9.33	+6.97	–2.36
Tear index, mNm^2/g	6.73±0.27	–10.85	–5.65	+5.20
Burst index, $kPam^2/g$	0.87±0.09	+33.59	+28.10	–5.49

± refers to standard deviation, (– reduction, + improvement)

The strain *Coprinopsis cinerea* HK-1 NFCCI-2032 grows well even at 47 °C, though the highest xylanase titre (695.8 IU/mL) is recorded at a substrate:moisture ratio of 1:3 after 7 days of incubation at 37 °C, pH 6.4, along with 0.541 IU/mL of poorly associated cellulase activity. The xylanase exhibits remarkable stability and retains 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 of 65, 75 and 85 °C, respectively, proving its alkali-thermo-tolerance for biobleaching.[48] Singh *et al*. observe that under optimum SSF conditions of incubation (period – 7 days, temperature – 37 °C, pH 6.4, carbon source – wheat bran, and organic nitrogen source – yeast extract), the strain SH-1NTCC 1163 of *Coprinellus disseminatus* yields xylanase, CMCase, and laccase activities of 727.78 IU/mL, 0.925 IU/mL and 0.640 U/mL, respectively, while strain SH-2 NTCC 1164 yields 227.99 IU/mL, 0.660 IU/mL and 0.742 IU/mL, respectively.[23] The crude cellulase-free xylanases (xylanase and CMCase activities of 30.32 and 0.32 IU/mL for MLK01 and 36.87 and 0.25 IU/mL for MLK07, respectively) are active over a wide pH range of 4 to 9.4, with the maximum relative xylanase activity (100%) at pH 7.5, MLK07 being active at a pH varying from 4 to 9.8, with the maximum relative xylanase activity (100%) at pH 8.0.[49]

CONCLUSION

The *C. cinereus* AT-1 MTCC 9695 produces maximum xylanase, cellulase and laccase activities of 698.75, 1.01 and 25.6±3.2 IU/mL, respectively, using wheat bran and yeast extract as carbon and nitrogen sources, at an incubation period of 7 days, incubation temperature of 37 °C, pH 6.4 and solid substrate to NSS ratio of 1:3. Crude xylanase enzyme produced by *C. cinereus* AT-1 MTCC 9695 is mesophillic in nature, as it shows maximum xylanase activity at 55 °C and pH 6.4. The molecular weights of these proteins are found to be of 50 and 83 kDa, respectively, for fungal strain *C. cinereus* AT-1 MTCC 9695. Therefore, xylanase isolated from strain *C. cinereus* AT-1 belongs to the high molecular weight category. The pH of SOP varies from 5.0 to 7.5, depending upon types of sizing (acidic or

neutral/alkaline). Therefore, the pH and temperature of diluted pulp slurry after deinking are about 6.0-7.0 and 45-55 °C, respectively, which are suitable for xylanase treatment before deinking. Crude xylanase acts as extender to cellulase-based deinking and with cellulase, it not only reduces the ERIC value and dirt counts, but also improves brightness, pulp viscosity, D_B and D_E significantly, compared to deinking with cellulase alone. The effect of xylanase with cellulase is observed in the form of increased total solids, BOD and COD in the combined effluent, compared to cellulase-based deinking. Therefore, it can be concluded that crude xylanase from *C. cinereus* AT-1 MTCC 9695 is suitable for biodeinking of SOP and the highest deinking efficiency is obtained using the cellulase–xylanase system.

ABBREVIATIONS

ABTS	2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
CMCase	Carboxylmethylcellulase
CMC	Carboxymethylcellulose
COD	Chemical oxygen demand
CSF	Canadian standard freeness
D_E	Deinkability factor based on ERIC
D_B	Deinkability factor based on brightness
DNS	3,5-dinitrosalicylic acid
ERIC	Effective residual ink concentration
FPase	Filter paper activity assay for cellulases
IU	International unit
NSS	Neutral salt solution
PDA	Potato dextrose agar
SDS–PAGE	Sodium-dodecylsulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOP	Sorted office paper
SmF	Submerged fermentation
SSF	Solid-state fermentation
TEMED	Ammonium persulphate and N, N, N', N'-tetramethylethylenediamine
w/v	Weight by volume

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