# NOVEL SOURCES OF FUNGAL CELLULASES FOR EFFICIENT DEINKING OF COMPOSITE PAPER WASTE

Rohit Soni, Asiya Nazir, B. S. Chadha,* and H. S. Saini

Twenty thermophilic/thermotolerant fungal strains were isolated from compositing soils and screened for production of different enzymes (Endoglucanases, β-glucosidase, Fpase and xylanases) to assess their deinking efficiency. Three isolates, *Aspergillus* sp. AMA, *Aspergillus terreus* AN1, and *Myceliophthora fergusii* T4I, identified on the basis of morphological and sequencing of amplified ITS1-5.8S-ITS2 rDNA region, showed significant deinking of composite waste paper (70% magazine and 30% Xerox copier/ laser print paper waste) as well as improved properties (brightness, tensile strength, tear index) of recycled paper sheets. The chosen strains *Aspergillus* sp. AMA, *Aspergillus terreus* AN1 and *Myceliophthora* fergusii T4I, showed 53, 52.7, and 40.32% deinking with increase in brightness by 4.32, 3.56, and 3.01 % ISO, respectively. These cultures were found to produce multiple endoglucanases and were characterized to lack a cellulose binding module (CBD), which may be responsible for their better deinking efficiency.

*Keywords: Thermophilic fungi; Screening; Cellulases; Deinking; Composite paper waste; EG without CBD*

*Contact information: Department of Microbiology, Guru Nanak Dev University, Amritsar- India; the first two authors contributed equally. *Corresponding author: chadhabs@yahoo.com*

## INTRODUCTION

Shortage of forest-based raw materials and problems in processing agro-residues are the major constraints in growth of production from the paper industry. Stringent environmental protection guidelines have also forced paper mills to go for deinking of the waste paper. The waste fiber used to make high-grade recycled paper consists mainly of magazine waste (OMG), mixed office waste (MOW), and old newspaper (ONP). Due to lack of any organized sector for waste paper collection in India, imported paper waste, comprising mainly OMG and MOW, constitutes the main ingredient used for preparing recycled paper pulp (Nichat 2003). These waste furnishes differ in the type of ink formulations and chemical composition. The deinking of composite waste comprising OMG and MOW is suitable for producing newsprint as well as high grade paper because it has longer and brighter fibers when compared to ONP. However, the presence of clay coatings and glue binders in OMG and hard-to-remove thermoplastic copolymer inks in MOW makes the conventional chemical deinking processes expensive and having high potential for environmental damage (Prasad et al 1992; Watson 1988). Therefore, enzymatic deinking is now being considered as a suitable alternate option for replacing some of the deinking chemicals. Workers in the recent past have suggested that microbial enzymes such as cellulase (endoglucanase and β glucosidase), xylanases (Morkbak and

Zimmerman 1998; Elegir et al. 2000; Sreenath et al. 1996; Qin et al. 1998; Viestures and Leitte 1999), esterases, and lipases have important role to play in biological deinking. The enzymatic treatment favors ink detachment from fibers without discharge of pollutants, thus contributing to environmental compatibility. The effect of cellulase in facilitating ink detachment during deinking of xerographic and laser printed-paper has been shown using preparations containing mixed enzyme (endoglucanase, cellobiohydrolase, β-glucosidase and xylanases) and mono-component activities (Prasad et al. 1992; Jeffries et al. 1994). On the other hand, removal of oil-based inks from magazine wastes is facilitated by addition of lipases and esterases. The cellulases bind and alter fiber surface or bonds in vicinity of ink particles resulting in removal of small fibers from the surface of ink particles, thus altering the relative hydrophobicity of the toner particles and reducing the hydrodynamic drag, which facilitate their removal during floatation step (Jeffries et al. 1994).

This study was designed to identify and evaluate novel sources of enzymes from diverse thermophilic/thermotolerant fungal isolates for biological deinking of composite paper furnish comprising of OMG and MOW. An extensive screening of these strains resulted in selection of *Aspergillus* sp. AMA, *Aspergillus terreus* $AN_1$, and *Myceliophthora fergusii* $T4_i$ as suitable microbial sources of enzymes, for effective deinking.

## MATERIAL AND METHODS

### Isolation of Fungal Cultures

Twenty thermophilic/thermotolerant strains were isolated from composting materials/ soils collected from different regions of India (Jammu and Kashmir, Amritsar, and Ahmedabad). These cultures were grown at 45°C on yeast starch agar (YpSs, pH 7.0) of the following composition (%; w/v): starch 1.5, yeast extract 0.4, $KH_2PO_4$ 0.23, $K_2HPO_4$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.05, citric acid 0.057, and agar 2.0 (Cooney and Emerson 1964) and maintained on the same medium at 4°C. The strains were screened for production of endoglucanase (EG), avicel adsorbable endoglucanase activity (AAEG), β glucosidase, Fpase, and xylanase; in a few selected strains cellobiohydrolase and esterases activities were also assayed. The resultant extracts were used for deinking of composite waste paper. Three strains showing efficient deinking were identified morphologically, microscopically, and on the basis of rDNA sequence of ITS1-5.8 S-ITS2 region.

### Extraction of DNA

The DNA was extracted from 40 mg of lyophilized mycelium (ground to fine powder) that was suspended in 550 µl of extraction buffer (1 M Tris HCl pH 8.0, 4M NaCl, 250mM EDTA, 1% β- Mercaptoethanol, 10% sodium dodecylsulfate, SDS) and 300 µl of equilibrated phenol. Upon homogenization, the tubes were incubated for 15 minutes at 65°C. The DNA in the aqueous phase was purified with repeated extractions using equal volumes of saturated phenol chloroform iso-amyl alcohol (PCI) mixture (25:24:1). The resultant DNA was precipitated with 9 parts of ice-cold isopropyl alcohol

and 1 part of sodium acetate (3M; pH 8.0). The tubes then were kept at -20°C for 2 h, followed by centrifugation for 15 minutes at 10,000 rpm. The resultant DNA pellets were rinsed with 70% ethanol, air dried, suspended in 50µl of sterilized double distilled water and stored at 4°C until use.

## PCR Amplification of ITS and 18S Region

The ITS1, ITS2 and the intervening 5.8S coding rDNA was amplified by PCR using ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTAT-TGATATGC 3') primer pair (White *et al*. 1990). Amplification reactions mixture (50µl) contained 25µl of PCR mix (Genei, Bangalore, India), 2.5µl of DMSO, 100 pmoles of each primer and 100 ng of DNA template. Thermal cycling consisted of initial denaturation of 4 minutes at 95°C, followed by 30 cycles, denaturation step at 94°C for 50 seconds, annealing step at 51°C for 1 minute, and primer extension at 72°C for 1 minute, followed by final extension step for 10 minutes at 72°C. Amplification products were electrophoretically resolved in 1.4% (w/v) agarose gel containing ethidium bromide, using 1X TAE buffer at 70 V.

## Solid Substrate Culturing for Enzyme Production

Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained ground rice straw as a carbon source (5 g) and basal medium (15 ml) of the following composition: $KH_2PO_4$ 0.4%, $CH_3COONH_4$ 0.45%, and $(NH_4)_2SO_4$ 1.3% (pH, 7.0), which was inoculated with spore suspension (2 ml; $10^7$ spores/ml), prepared from seven days old culture grown on agar plates. The flasks were incubated for five days at 45 $^oC$. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50mM pH 6.0) to the flasks and kept at 45 $^oC$ for 1h under mild shaking. The resultant slurry was filtered through muslin cloth and centrifuged at 8800 x g for 10 min, and the extracts were used for enzymatic assay.

## Enzymatic Assay

Endoglucanase (1, 4- β-glucan-4-glucanohydrolase, EC 3.2.1.4) and xylanase (endol, 4 β-D- xylanase, EC 3.2.1.8) activities were determined using 1% CM-cellulose and 1% Birch wood xylan, prepared in sodium citrate buffer (50 mM, pH 6.0), respectively. The reaction mixture containing equal amounts of suitably diluted enzyme and substrate was incubated at 50$^o$ C for 10 min and 5 min, respectively. The reaction was stopped by addition of DNS followed by boiling (Miller 1959); the colour developed was read at 540 nm using Novaspec II spectrophotometer (Pharmacia). The amounts of released sugar were quantified using glucose and xylose standards, respectively.

The avicel absorbable activity (AAEG) was assayed as described by Arifoglu and Ogel. (2000). The reaction mixture containing 0.5 ml of sodium acetate buffer (25 mM, pH 5.0), 0.5 ml of culture supernatant and 100 mg of avicel was kept at 4$^o$C for 1 h. After centrifugation, the residual EG activity in the supernatant was measured by using CM-cellulose (1%). AAEG was measured indirectly by subtracting avicel non-adsorb able EG activity from total EG activity. Total cellulose activity (Fpase) was measured by using a Whatman No. 1 filter paper strip (1x 6 cm) as substrate (Wood and Bhat 1998).

β-Glucosidase (β -D- glucosido- glucanohydrolase, EC 3.2.1.21) and cellobiohydrolase (EC 3.2.1.91) were assayed using *p*-nitro phenyl- β -D- gluco pyranoside (pNPG) and *p*-nitro phenyl- β -D- cellobioside, respectively, in a micro-titre plate based method (Parry et al. 2001). Appropriately diluted enzyme (25 µl) was mixed with 50 µl of sodium acetate buffer (50 mM, pH 5.0). The reaction was initiated by adding 25 µl of pNPG/pNPC (10mM) and incubated at 50 $^{o}$C for 30 min; the reaction was terminated by adding 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8), and the developed yellow color was read at 405 nm using an ELISA Reader (MULTISKAN; Lab system). One unit of β-glucosidase/ cellobiohydrolase activity was expressed as the amount of enzyme required to release 1 µmole of pNP per minute under assay conditions. For assay of esterase activity the above method was used, except that pNP acetate (1 mM) was used as substrate (Ghatora et al. 2006). The enzyme activities were expressed as units per gram dry weight substrate.

## Characterization of Crude Endoglucanase

Endoglucanase of the selected strains *Aspergillus* sp. AMA, *Aspergillus terreus* $AN_1$ and *Myceliophthora fergusii* $T4_i$ was characterized for temperature and pH optima and thermostability. The effect of temperature on the endoglucanase activity was analyzed between 30$^{o}$C and 90$^{o}$C at pH 5.0. The optimum pH for enzyme activity was determined using 0.05M buffers ranging from pH 3.0 to 10.0 (citrate buffer (pH 3-6), sodium phosphate buffer (pH 7-8) and glycine- NaOH buffer (pH 9-10)) at optimal temperature previously determined for each enzyme. The stability of endoglucanase was monitored at optimal pH and temperature for 1h and residual activities were assayed.

Strains selected on the basis of efficient deinking of waste paper were further characterized for presence of multiple endoglucanase by developing zymogram. Native polyacrylamide gel electrophoresis (PAGE) 10% with 1% resolving gel was used to resolve the desalted and concentrated enzyme samples (70 µg). After electrophoresis the gels were incubated for 15 min in 0.05 M sodium acetate buffer (pH 5.0) and overlaid on polyacrylamide gel containing CMC (0.5%,w/v) for 2 h at 50$^{o}$C. The overlay gel was removed and stained with 0.2% Congo Red. Bands corresponding to EG appeared as clear zone against a dark background after de-staining with 1M NaCl followed by treatment with 10% (v/v) acetic acid solution.

## Deinking Experiments

Composite paper waste comprising of 70% OMG waste and 30% MOW was disintegrated in laboratory pulper (Universal) for 10 min at 4% consistency using tap water. The disintegrated pulp was recovered by dewatering through 200 – mesh wire and oven dried. The resultant pulp was suspended in sodium citrate buffer pH 6.0 (10% consistency), and enzymatic treatment was carried out using an enzyme dose of 50 units/100 g of oven dried pulp at 50 $^{o}$ C. To inactivate the enzyme, the pulp suspension was boiled for 10 min. Finally the pulp was washed with tap water through 200-mesh wire and subjected to flotation (Lamort type original Kowaleshwski Floatation cell; capacity 25 Litres equipped with aerator operating at speed of 2500 rpm (model UEC-2026) by Universal Engineering Corporation , Saharanpur, India) for 20 min in presence of surfactant (0.1% Tween 80) and (0.1% $CaCl_2$) as flotation aids. Control assays without

enzyme treatment were carried out under identical conditions. After flotation, handsheets were prepared (TAPPI method T 205 sp-02) from enzyme-treated, as well as control pulp, using a semi-automatic handsheet maker (Universal Engineering Corporation, Saharanpur, India). The resultant handsheets were analyzed using an ELREPHO 70 image analyzer (Lorentzen and Wettre- Elrepho, Sweden). The brightness (TAPPI, T 452 om-02) and percent residual ink count in the handsheets was measured using the image analyzer at six different places on each handsheet, and results were expressed as mean values. The handsheets were also analyzed for properties like breaking strength, burst index, and tear index according to standard TAPPI methods (Marques et al. 2003).

## RESULTS

### Screening of Fungal Isolates for Production of Cellulases and Xylanase

Twenty fungal strains were isolated from soil collected from different regions of North India (Jammu and Kashmir, Amritsar, and Ahmedabad). These isolates, on the basis of morphological and microscopic examination of spore arrangements, were identified as *Myceliophthora* sp. strains, (V2A2, MYC and T4I), *Corynascus* sp. (JHG), *Humicola insolens* (J), *Malbranchea flava* (MF), *Melanocarpus* sp. (MEL), *Aspergillus* sp. (LC11, AMA, A6 and AN1), *Torula* sp. (ASS2, T22, T32 and AN6), *Emericella nidulans* (F), *Penicillium* sp. (CD2W3), *Chaetomium* sp. (1CHP), and an unidentified strain CS12. The molecular characterization of the strains showed that *Myceliophthora* sp. (MYC),. *Corynascus* sp. (JHG), and *Myceliophthora fergusii* (T4I) formed a distinct clade, whereas, *Malbranchea flava* (MF) had a distinct phylogenetic origin. The strains of *Aspergillus* sp. (AMA) and *A. terreus,* though, belonging to the same genus, were phylogenetically distinct and were supported by lower bootstrap values (Fig. 1). The strains were grown on solidified rice straw based culture medium at 45$^{o}$C and studied for production of different enzymatic activities which included, endoglucanase (EG), avicel absorbable endoglucanase (AAEG) , Fpase, β-glucosidase, and xylanase.

The results in Table 1 show that of the strains included in study, *Melanocarpus* sp. (MEL), produced maximal amount of endoglucanase (115 units/g DW substrate), followed by *Aspergillus* sp. strain AMA (98.5 units/g DW substrate) and *A. terreus* AN1 (77.0 units/g DW substrate). The culture filtrates of *Aspergillus* sp. (AMA), *A. terreus* (AN1), and *M. fergusii* (T4I), did not show any AAEG activity under the given culture conditions. On the other hand *H. insolens* (J) showed maximal AAEG activity corresponding to 76.8 % of the total EG activity. Similarly higher fractions of AAEG activity was also observed for *Corynascus* sp. (JHG) and *Torula* sp.(AN6).

The strain of *Melanocarpus sp*. (MEL) also produced high levels of FPase (11.65 units/g DW substrate) followed by *Aspergillus* sp. (AMA) which produced 4.5 units/g DW substrate of activity in addition to high levels of β-glucosidase activity (250 units/g DW substrate). However, the highest β-glucosidase activity was recorded in *A. terreus* AN1 (500 units/g DW substrate), followed by *Penicillium* sp. CD2W3 (110 units/g) and *Aspergillus* sp. LC11 (108.2 units/g). High level of xylanase was achieved in *Malbranchea sp.* (9000 units/g DW substrate) followed by *Penicillium* sp. CD2W3 that showed (6011 U/g DW substrate). The titers of xylanase in *Aspergillus* sp. AMA (2782

units/ g DW substrate) and *A. terreus* (2580 units/ g DW substrate) were also high. In addition the observed levels of esterase activity in *Aspergillus* sp. *(AMA), Aspergillus terreus* (AN1) and *Myceliophthora fergusii* (T4I) were 86.7, 78.7, and 72.1 (units/ g DW substrate), respectively.

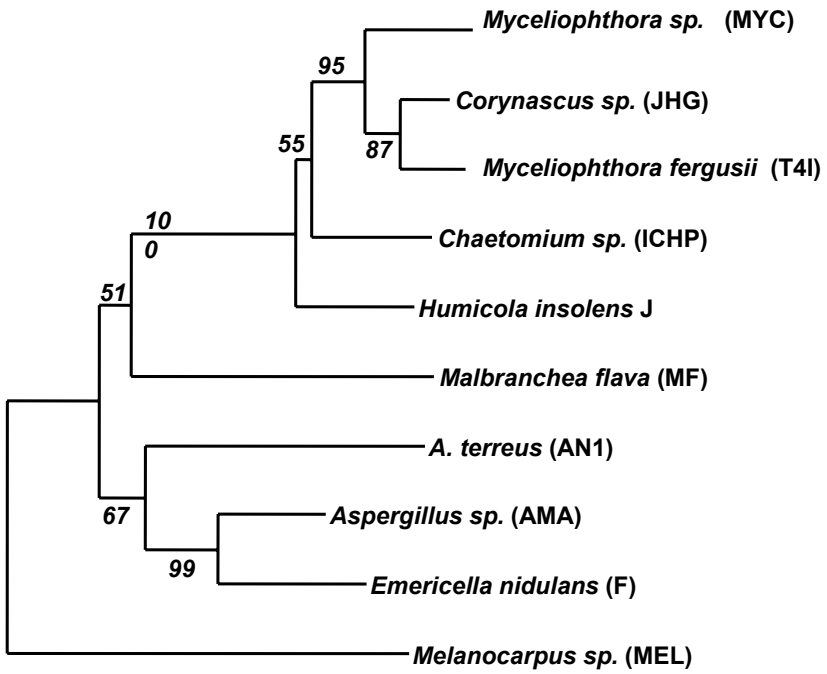



**Fig 1.** Diagram showing the dendrogram of fungal strains used in the deinking experiment. The strains were characterized on the basis of their ITS1-5.8S-ITS2 sequences and identified, in addition to morphology, on the basis of the alignment of the sequences from NCBI databases. The values at the nodes are bootstrap values.

## Deinking Experiments

Table 2 shows results of deinking composite waste comprised of 70% OMG and 30% MOW. The handsheets were prepared with the composite pulp treated with different multi-component enzymes at a dosage of 0.5 units CMCase (EG)/ g pulp. Two parameters i.e., percent residual ink and brightness (ISO), were considered during screening of the potential of these enzymes for biodeinking. The results (Table 2) showed that treatment of pulp with enzyme preparations from *Corynascus* sp. (JHG), *A. terreus* (AN1), *Torula* sp. (AN6), *Myceliophthora* sp. (V2A2), *M. fergusii* (T4i), *Aspergillus* sp. AMA, and *Malbranchea flava* (MF), yielded improvements in brightness. However, all the other preparations resulted in decrease in percent ISO brightness as compared to the control. All enzymes included in study, however, resulted in decrease in percent residual ink ranging between 5 to 53 %, when compared to the control. Maximal deinking efficiency was observed in *Aspergillus* sp. AMA, which showed up to 53% reduction in ink, followed by *A. terreus* AN1 (52.7%) and *M. fergusii* (T4I), showing 40.5 % reduction, with improved brightness of the resultant hand sheets by 4.32, 3.56 and 3.01 % ISO, respectively.

**Table 1.** Production of Cellulases and Xylanase by Thermophilic / Thermotolerant Fungal Isolates

| Location | Strains | CMC-ase (Units/g) | Fpase (Units/g) | β-gluco-sidase (Units/g) | Avicel adsorbable activity (Units/g) | Xylanase (Units/g) |
|---|---|---|---|---|---|---|
| Jammu | *Aspergillus* sp. (LC11) | 48.0 | 1.99 | 108.2 | 3.7 | 192 |
| | *Corynascus* sp. (JHG) | 51.2 | 0.56 | 11.4 | 24.8 | 358 |
| | *Aspergillus terreus* (AN1) | 77.0 | 4.65 | 500 | 0 | 2580 |
| | *Torula* sp. (AN6) | 28.0 | 0.142 | 22.0 | 12.9 | 82.0 |
| | *Myceliophthora* sp (V2A2) | 31.3 | 0.627 | 20.13 | 9.2 | 590.2 |
| | *Torula* sp. (T22) | 11.4 | 0.20 | 41.0 | 2.9 | 170 |
| | *Torula* sp. (T32) | 26.0 | 0.96 | 9.8 | 2.5 | 206 |
| Amritsar | *Torula* sp. (ASS2) | 42.5 | 0.99 | 63.3 | 14.3 | 260.9 |
| | *Myceliophthora fergusii* (T4I) | 36.7 | 2.29 | 53.5 | 0 | 884.7 |
| | *Aspergillus* sp. (AMA) | 98.5 | 4.0 | 250 | 0 | 2782 |
| | *Melanocarpus* (MEL) | 115 | 11.65 | 27.4 | 23 | 1990 |
| | *Aspergillus* sp. (A6) | 47.9 | 0.85 | 24.8 | 18.6 | 225 |
| | *Emericella nidulans*. (F) | 21.5 | 2.4 | 77.0 | 10.2 | 329.5 |
| | *Myceliophthora* sp. (MYC) | 35.0 | 2.44 | 7.48 | 5.0 | 900.2 |
| | *Penicillium* sp. (CD2W3) | 42.0 | 2.33 | 110 | 1.0 | 6100 |
| | *Malbranchea flava* (MF) | 21.4 | 2.16 | 22.3 | ND | 9000 |
| | *Penicillium* sp. (T4K) | 18.0 | .72 | 24.0 | | 43.0 |
| Ahmed-abad | Unidentified (CS12) | 54.0 | 1.9 | 1.82 | ND | 302 |
| | *Chaetomium* sp.(1 CHP) | 54.5 | 1.3 | 3.6 | ND | 285 |
| | *Humicola insolens* (J) | 33.3 | 1.99 | 55.8 | 25.6 | 271.9 |

Activities expressed as units/g DW substrate; ND Not Determined

The handsheets prepared from pulp treated with selected enzyme extracts of *Aspergillus* sp. AMA, *Aspergillus terreus* $AN_1$, and *Myceliophthora fergusii* T4I were evaluated for optical as well as physical and mechanical strength in order to measure the influence of enzyme on deinking (Table 3). The results showed an appreciable increase in burst index and tensile strength of the handsheets prepared after enzymatic treatment. When compared to the control, maximal increases in burst index (74%) and tensile index (52%) were observed in the recycled fibre treated with the enzyme of *A. terreus*. On the other hand, a marked improvement in tear index (9.7%) was observed in the handsheets prepared of enzyme from *M. fergusii* (T4I). A negligible improvement in tear index was observed in hand sheets prepared with fibre treated with *A. terreus* (AN1) and *Aspergillus* sp. (AMA) enzymes.

**Table 2.** Effect of Enzymatic Treatment on Residual Ink and Brightness

| Sr. No. | Organism | Residual ink | | Brightness | |
|---|---|---|---|---|---|
| 1. | Control | 63.62 | ±1.34 | 81.96 | ±0.84 |
| 2. | Chemical deinking* | 30.77 | ± 2.80 | 81.13 | ± 0.42 |
| 3. | *Aspergillus* sp. (LC11) | 45.96 | ±0.28 | 78.98 | ±0.12 |
| 4. | *Corynascus s*p. JHG | 60.29 | ±0.72 | 84.30 | ±0.20 |
| 5. | *Aspergillus* sp (AN1) | 30.08 | ±0.33 | 85.52 | ±0.05 |
| 6. | *Torula* sp. (AN6) | 57.37 | ±0.002 | 80.00 | ±0.005 |
| 7. | *Myceliophthora* sp (V2A2) | 56.32 | ±1.51 | 84.97 | ±0.24 |
| 8. | *Torula* sp. (T22) | 54.24 | ±0.300 | 77.76 | ±0.13 |
| 9. | *Torula* sp. (T32) | 66.39 | ±0.49 | 76.05 | ±0.20 |
| 10. | *Torula* sp. (ASS2) | 45.84 | ±0.36 | 79.46 | ±0.10 |
| 11. | *Myceliophthora fergusii* (T4I) | 37.84 | ±0.54 | 82.69 | ±0.08 |
| 12. | *Aspergillus* sp. (AMA) | 29.89 | ±0.23 | 86.28 | ±0.05 |
| 13. | *Melanocarpus* (MEL) | 60.63 | ±0.58 | 78.90 | ±0.18 |
| 14. | *Aspergillus* sp. (A6) | 60.65 | ±0.43 | 77.02 | ±0.19 |
| 15. | *Emericella nidulans.* (F) | 63.33 | ±4.94 | 79.11 | ±0.36 |
| 16. | *Myceliophthora* sp. (MYC) | 56.43 | ±3.39 | 79.34 | ±0.45 |
| 17. | *Penicillium* sp. (Cd2W3) | 59.25 | ±2.62 | 81.90 | ±0.025 |
| 18. | *Malbranchea flava* (MF) | 58.30 | ±0.59 | 82.22 | ±0.36 |
| 19. | *Penicillium* sp. (T4K) | 58.04 | ±0.02 | 80.04 | ±0.01 |
| 20. | Unidentified (CS12) | 66.14 | ±0.73 | 78.61 | ±0.22 |
| 21. | *Chaetomium* sp. (ICHP) | 52.96 | ±0.93 | 78.80 | ±0.09 |
| 22. | *Humicola insolens* (J) | 66.57 | ±1.5 | 78.24 | ±0.15 |

*Chemical deinking was carried out with Sodium silicate 2%; Sodium hydroxide 2% & $H_2O_2$ 1% instead of enzyme.

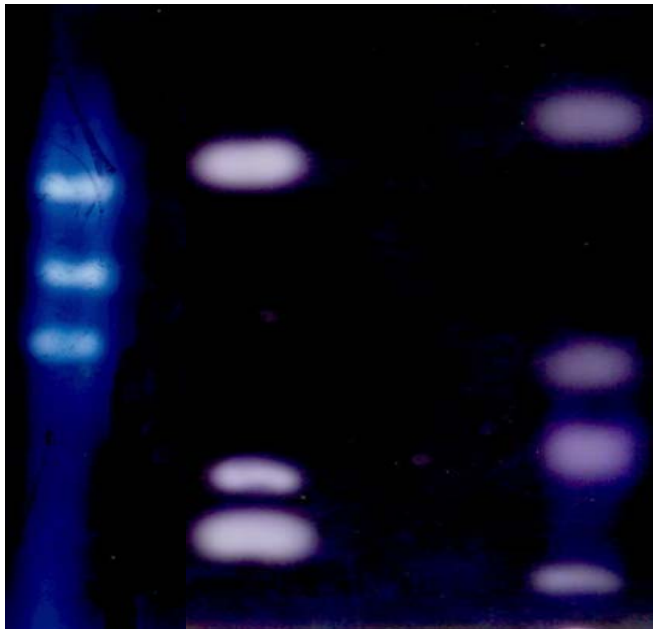
**Table 3.** Effect of the Enzymatic Treatment on Physical and Mechanical Strength Properties

| | Deinking efficiency (%) | Brightness (% ISO) | Burst index (KPa $m^2$/g) | Tensile index (Nm/g) | Tear index (m$Nm^2$/g) |
|---|---|---|---|---|---|
| Control pulp | - | 81.91 | 2.34 | 38.5 | 9.3 |
| *Aspergillus* sp. (AMA) | 53 | 86.28 | 3.99 | 56.3 | 9.5 |
| *A. terreus* | 52.7 | 85.52 | 4.09 | 58.80 | 9.84 |
| *M. fergusii* | 40.5 | 82.69 | 3.99 | 58.57 | 10.21 |

Composite paper waste used for preparing recycled sheets

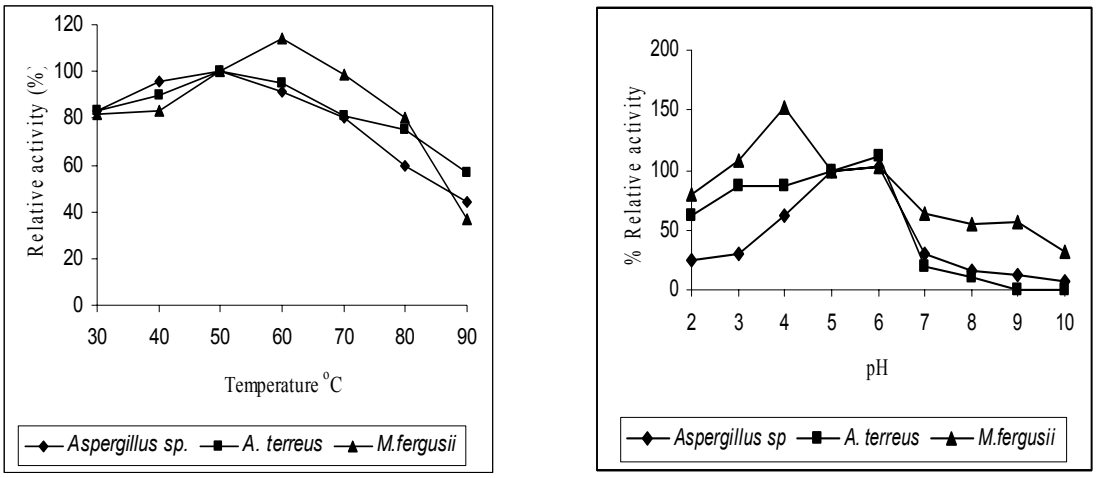
## Endoglucanases of Selected Isolates

The selected strains of *Aspergillus* sp. (AMA), *A. terreus* (AN1), and *M. fergusii* (T4I) were further characterized for multiplicity of the endoglucanases (EG). The enzyme extracts were resolved on PAGE, and zymogram for EG were developed. The results in Fig. 2 showed the presence of 4 endoglucanases of different electrophoretic mobility in *A. terreus* (AN1) and three isoforms in extracts of Aspergillus sp. (AMA) and *M. fergusii* (T4I). The EG of strains of *Aspergillus* sp. (AMA) and *A. terreus* (AN1) exhibited optimal activity in range at 50°C, whereas, *M. fergusii* (T4I) was optimally active at 60°C. The enzymes from all three strains retained 100% activity up to pH 6.0 (Fig. 3).



(a) ( b) (c)

**Figure 2.** Zymogram showing multiplicity of endoglucanase in (a) *M. fergusii* (T4I) b) *Aspergillus* sp. (AMA) c) *A. terreus* (AN1) resolved by PAGE.




(a) (b)

**Figure 3.** Effect of temperature (a) and pH (b) on the cellulase activity of *Aspergillus* sp. AMA, *Aspergillus terreus* AN1 and *Myceliophthora fergusii* T4I.

## DISCUSSION

The biological option, using cellulases and esterases, is now a well-documented approach for deinking and recycling of waste paper. Many research papers and a few patents in recent past have been published in this regard. Most of the initial studies on deinking have been carried out with a view to replace chemicals with enzymes. The

cellulases that have mainly been tested are the commercially available sources of multi/mono-component enzyme preparations of *Trichoderma reseei*, *H. insolens* supplied by Novo-Nordisk, IOGEN, Genencor, etc (Pala *et al*., 2004; Jefferies et al. 1994). In addition a few of the recent studies have also tested cellulases (with xylanase activities) from the wild type isolates of *A. terreus, Aspergillus* L22, *Trichoderma pseudokoningii, Gleophyllum* sp. *,* O*rpinomyces* sp. *, Fusarium* sp., etc. (Marques et al. 2003; Gubitz et al. 1998; Geng and Li 2003; Vyas and Lachke 2003).

These enzyme preparations contain mixtures of cell wall degrading enzymes, with endoglucanase (EG) as an important component that randomly chops off the pulp fiber on which ink is sticking. However, most of the cellulase preparations contain multiple forms of EG, which may be classified in the same or different cellulase families. The endoglucanase on the basis of amino acid sequence/hydrophobic cluster analysis can be classified into different families (Henrissat et al. 1988). These EG on the basis of affinity for crystalline cellulose, can be further subdivided into those that contain Cellulose Binding Module (CBM) and have specific mechanism of action that is different from those that lack CBM. EG with CBM containing a catalytic domain that is joined to CBD by a linker rich in threonine, serine, and proline (Harrison et al. 1998) have been reported both in endoglucanases and cellobiohydrolases of *Trichoderma reesei, Humicola insolens,* etc. On the other hand, a few of the cellulases classified in families 5, 7, and 12 are reported to lack CBD (Lonsky and Negri 2003). Family 5 endoglucanases from *Bacillus* sp. strain KSM-64, and *A. aceulatus*, family 7 cellulase from *H. insolens* and *M. thermophila* as well as family 12 cellulase from *T. reesei,* which lack CBD are preferred endoglucanases for deinking and textile industry (Lonsky and Negri 2003). Similarly, cellulases Cel B and Cel E from anaerobic fungus *Orpinomyces* sp. that lack CBD have been found suitable for deinking of MOW (Geng and Li 2003). During extensive screening we identified *Aspergillus* sp. (AMA), *A. terreus* (AN1), and *M. fergusii* (T4I) as the strains that produced multiple isoforms of EG and did not show avicel adsorbable endoglucanase activity (AAEG), indicating an apparent lack of CBD. Furthermore, enzymatic deinking with these strains was found to be most efficient, as compared to other sources. Moreover, during purification we found that the endoglucanases bound tightly to the phenyl sepharose column, indicating the hydrophobic nature of enzyme. It has been suggested that removal of fibrous material from toners particles would increase hydrophobicity and thereby facilitate separation during flotation (Jefferies et al. 1994).

The next important thing considered in this paper was to isolate enzymatic sources that exhibit high enzyme titres in the culture extracts for process economics. The screening of fungal isolates for production, of cellulases, and hemicellulase, by Solid Substrate Fermentation (SSF), using rice straw as a carbon source, was employed. We have previously shown that rice straw, owing to its heterogeneity of composition, is a suitable source of carbon for producing plant cell wall degrading enzymes by *Myceliophthora* sp., *Melanocarpus* sp. and *Scytilidium thermophilum* (Badhan et al. 2007; Jatinder et al. 2006 a & b). The screening showed *Aspergillus* sp. and *A. terreus*, produced high titres of EG, the observed FPase and β glucosidase activities were also appreciably higher, and the ratio of FPase: β glucosidase was around 70 to 100. The enzyme preparations also contained good levels of xylanases and esterases. It has been shown in a recent patent (Yang et al. 2004) that enzymes with FPase to β glucosidase in

ratio of 1: 100 are efficient for deinking of MOW. In a previous study the positive role of xylanases from *T. lanuginosus* as an additive to endoglucanases was shown to enhance removal of toners from laser printed ink (Gübitz et al. 1998).

The observed enzyme titres in selected isolate of *A. terreus* AN1 in the present study was appreciably higher than that reported in the *A. terreus* isolate CCMI 498 by Marques and co-workers (2003), which may be attributed to the fact that geographically distinct strains show marked variations in the enzyme titres and hence there is need to isolate and screen geographically distinct strains (Sonia et al. 2005).

Following the enzymatic deinking protocol as suggested by Pala and co-workers (2004), we found that deinking of the composite waste by selected enzymes from *A. terreus* (AN1), and *Aspergillus* sp. (AMA) were efficient for deinking. The observed percent deinking were apparently higher as compared to those observed for different commercial enzymes for deinking of non-impact ink printed paper (Pala et al. 2004), however, it may be noted that ink removal from non-impact printing is considered more difficult as compared to other printed paper types. The reason for the observed efficient removal by selected enzymes in this study may be ascribed to either reduction in ink particle size or modification of the ink/surface properties by these enzymes. Moreover, it has also been observed that the removal of the toners with EG without CBD is more efficient in presence of surfactant, as compared to the one with CBD, as these cellulases bind tightly to the cellulose fibre and their removal by non-ionic surfactants is relatively difficult (Lonsky and Negri 2003). The observed reduction in the residual ink also correlated well with the improved brightness of the handsheets. The observed improvement in percent ISO brightness of paper furnishes treated with *Aspergillus* sp. AMA enzyme is among one of the highest reported (Vyas and Lachke 2003). However, no such correlation between deinking and brightness was observed as reported previously (Jefferies et al. 1994). The presence of the pigments in the crude enzyme preparations may some time hinder the improvement in brightness of the paper, and therefore use of partially purified enzymes is the suggested approach.

An appreciable improvement in physical properties of the handsheets prepared with selected enzymes in this study was observed. In fact the observed levels of improvement were much higher as compared to those reported by Pala et al. (2004). However, the observed increases in tensile index and burst index may be attributed to inter-fiber bonding that are characteristic of furnishes from OMG, as used in the present study. A recent patent reported enzymatically deinked paper with 50 to 150% increased tensile index (Lonsky and Negri 2003). Our study showed that rigorous and systematic screening of suitable microbial sources of enzymes can be useful in developing efficient deinking technologies. Further work on the purification of mono-component enzymes and their role in deinking from *Aspergillus terreus* $AN_1$ and *Aspergillus* sp, AMA, as well as improvement of strains by protoplast fusion, cloning and over expression, etc. for achieving higher enzyme titres, is in progress.

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