OPTIMIZATION OF CELLULASE PRODUCTION UNDER SOLID-STATE FERMENTATION BY *ASPERGILLUS FLAVUS* (AT-2) AND *ASPERGILLUS NIGER* (AT-3) AND ITS IMPACT ON STICKIES AND INK PARTICLE SIZE OF SORTED OFFICE PAPER

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Out of 12 fungal strains, two fungal strains, namely *Aspergillus flavus* AT-2 and *Aspergillus niger* AT-3, were found to produce maximum cellulase production. The CMCase activities of *A. flavus* AT-2 and *A. niger* AT-3 under state solid fermentation (SSF) conditions were of 42.69 and 40.08%, respectively, which was higher than those obtained under submerged fermentation (SmF) conditions. A fungal inoculum dose of 5%, incubation period of 5 days, temperature and pH of 30 °C and 4.8 for *A. flavus* AT-2 and 35 °C and 5.3 for *A. niger* AT-3, respectively, solid substrate:moisture content 1:3, rice straw (treated) as carbon source, $(NH_4)_2SO_4$ + yeast extract as nitrogen source, KH_2PO_4 as phosphorus source, succinic acid as source of organic acid, Tween-80 as surfactant and cellobiose as source of soluble sugar might be taken as optimal for cellulase production for both fungal strains. The optimum CMCase (17.24 IU/mL), FPase (1.92 IU/mL), β-glucosidase (0.69 IU/mL) and xylanase activities (5.73 IU/mL) and fungal protein concentration (2.90 mg/L) for *A. flavus* AT-2 and CMCase (24.32 IU/mL), FPase (2.47 IU/mL), β-glucosidase (0.82 IU/mL) and xylanase activities (4.80 IU/mL) and protein concentration (3.07 mg/L) for *A. niger* AT-3 were taken as optimal under SSF conditions. *A. flavus* AT-2 and *A. niger* AT-3 were found active in the pH range of 4.0 to 7.5 and maximum cellulase activity was obtained at pH 5.0 for *A. flavus* AT-2 (17.31 IU/mL) and at pH 5.5 for *A. niger* AT-3 (24.50 IU/mL). The optimum temperature for the crude cellulase activities of both fungal strains (*A. flavus* AT-2, IU/mL and *A. niger* AT-3, IU/mL) was of 50 °C, and beyond it cellulase activities were found to decrease. Deinking of sorted office paper with cellulase reduced stickies level by 4%, compared to control and concoctions of different enzymes, i.e. cellulose, xylanase, amylase and lipase in different combinations, were capable of removing ink particles of all sizes from the pulp.

Keywords: *Aspergillus flavus* AT-2, *Aspergillus niger* AT-3, solid-state fermentation, cellulase activity, stickies, ink particle size

INTRODUCTION

Cellulases are currently the third largest industrial enzyme worldwide, by dollar volume, due to their wide applications in cotton processing, paper recycling, juice extraction, as detergent enzymes and animal feed additives. However, cellulases may reach the largest volume of industrial enzyme; if ethanol from lignocellulosic biomass through the enzymatic route becomes a major transportation fuel.[1] Cellulolytic enzymes are produced by a variety of bacteria, fungi, actinomycetes, aerobes and anaerobes. Each of these microorganisms can produce different kinds of cellulases that differ in their mode of action as well as properties like activity towards crystalline cellulose, activity and stability in acidic or alkaline pH. The most privileged sources for the production of cellulases are fungi, due to their higher enzyme yields and capacities to produce complete cellulase complex. The cellulase systems of the aerobic fungi, *Trichoderma reesei*, *T. viride*, *T. koningii Penicillium pinophilum*, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani*, *Talaromyces emersonii*, *Rhizopus oryzae*[2,3] *Humicola insolens*[4] and *Aspergillus niger*,[5] are well characterized.

Cellulases have a potential in the pulp and paper industry, e.g. in deinking to release ink

from fibre surfaces and in improving pulp drainage.[6] Within the forest industry, cellulases have been shown to be effective in decreasing the energy consumption of mechanical pulping.[7] Cellulases have been found to increase the alkali solubility of treated pulp and directly alkali soluble cellulose has been obtained with specific cellulase compositions.[8] This property can be utilized in developing new, environmentally benign processes for manufacturing cellulosic articles, such as films and fibres.

Solid-state fermentation on wheat bran based medium at 37 ^{o}C is considered as a favourable mode for growth and production of xylanases (499.60 IU/mL) by a newly isolated thermo-alkali-tolerant white rot fungus *Coprinellus disseminatus* SW-1 NTCC 1165 at pH 6.4.[9] Solid-state fermentation is 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 IU/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 ^{o}C and pH 6.4.[10] Cellulase from *Aspergillus niger* AT-3 ITCC 6325 shows CMCase, FPase and β-glucosidase activities of 25.12, 2.23 and 0.87 IU/mL, respectively, and protein concentration of 5.8 mg/mL.[11] α-amylase from *Aspergillus niger* produces an activity of 0.08 IU/mL/min with the substrate of wheat bran at 28 °C and pH 6.2 under solid-state fermentation conditions.[12] *Rhizopus oryzae* produces maximum lipase activity of 120 IU/mg at a pH of 7.5 and incubation period of 4 days under solid-state fermentation.[13]

The present study aims at isolating, screening and identifying the microorganisms (fungi) that are capable of producing extracellular cellulases. The optimization of various operating physico-chemical parameters was done to achieve higher cellulase activity from the screened strains. The cellulase was biochemically characterized to check its temperature and pH stability for its successful utilization in enzymatic deinking experiments.

EXPERIMENTAL

Isolation of microbial strains

The fungal strains were isolated from decaying cotton cloths and dead and decaying wood from Saharanpur (UP) and Roorkee (UK) located in the foothills of Shivalik hills in Northern India, by an enrichment technique (using wheat bran as carbon source). Purified cultures were maintained on PDA slants.

Preparation of inoculums

The culture of the PDA slant was first incubated at 30 °C for 24 h. The activated spores were removed and suspended in Tween-80 (0.01%, *v*/*v*) and 2 mL of spore suspension (2×10^6 spores per mL) was transferred into 50 mL of Mandels and Weber's modified pre-culture medium, containing 10 g/L glucose at pH 4.8.[14] The medium after inoculation was incubated at 30 °C for 24 h with continuous shaking (120 rpm) in an orbital shaker.

Enzyme production

Enzyme production was carried out under solid-state fermentation (SSF) and submerged fermentation (SmF). For SSF, a slurry of the fermentation medium, containing 5 g of wheat straw and 15 mL of NSS, was prepared in Erlenmeyer flasks (250 mL) and inoculated with fungal inoculums. The nutrient salt solution (NSS) prepared for the production of cellulases contained, as g/L, 2.1 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 $MgSO_4.7H_2O$, 0.3 $CaCl_2$, 0.00156 $MnSO_4.5H_2O$, 0.0014 $ZnSO_4.7H_2O$ and 0.0026 $CoCl_2.6H_2O$ with 2.0 mL/L Tween-80. The culture flasks were incubated at 30 °C for 5 days. SmF was carried out in 50 mL of basal medium, containing NSS with 1% cellulosic substrate (synthetic or lignocellulosic waste) as carbon source in Erlenmeyer flasks (250 mL). The medium was inoculated with 5% of the inoculums of fungal cultures, followed by incubation at 30 °C for 5 days in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm).

Extraction of enzymes

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

Enzyme assays and protein determination

CMCase activity was determined as described by Mandels.[15] The assay mixture, in a total volume of 2 mL, contained 0.5 mL of 1 mM of carboxyl methyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 mL of diluted crude enzyme. The mixture was incubated at 50 °C for 30 min. FPase activity was determined as described by Rajendran *et al.*[16] The assay mixture (total volume of 2 mL) contained 50 mg

of Whatman No.1 strip (1 × 6 cm) in 1 mL of 50 mM citrate buffer (pH 4.8) and 0.5 mL of diluted crude enzyme and was incubated at 50 °C for 30 min. β-glucosidase activity was estimated using p-nitrophenyl β-D glucopranoside (p-NPG) as substrate.[17] The assay mixture, in a total volume of 1 mL, contained 50 mM of substrate, 50 mM citrate buffer (pH 4.8) and 0.1 mL of diluted crude enzyme. The reaction mixture was incubated at 50 °C for 30 min. Following incubation, 2 mL of 4% sodium carbonate solution was added and the p-nitrophenol released was measured at 410 nm with a UV-Vis spectrophotometer. Xylanase activity was estimated by analysis of the xylose units released by DNS method.[18] 0.4 mL of 1% birchwood xylan solution was mixed with 1.6 mL of suitably diluted culture filtrate in 50 mM potassium phosphate buffer (pH 6.4) and incubated at 55 °C for 15 min. 0.3 mL of solution was taken from the incubated mixture in a test tube and 0.9 mL of DNS reagent was then added, boiled for 5 min. The reducing sugars released were measured optically at 540 nm with a UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 °C, using 3,5-dinitrosalicylic acid (DNS) reagent.[18] Protein concentration was estimated according to Lowry method,[19] with bovine serum albumin (BSA) as a standard.

Effect of various nutritional parameters and optimization of fermentation conditions

The effect of various nutritional parameters like carbon sources and nitrogen sources, inoculums, sugars, phosphorus, organic acids, and surfactants were investigated and optimization of incubation period, temperature, moisture level and pH level under SSF conditions was carried out in order to achieve maximal production of cellulases.

Bio-chemical characterization of crude cellulase

The optimum pH of enzymes was determined in the pH range of 4.0-7.5 by incubating the enzyme with buffers of different pH, *viz.* citrate buffer (pH 3-6), potassium-phosphate (pH 6.0-7.4) and sodium phosphate buffer (pH 7-8). The optimum temperature of the enzyme was determined by incubating the crude enzyme preparations at temperatures ranging from 50-80 °C for 30 min.

Electrophoresis and zymogram analysis

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities *in situ*, as described by Reyes *et al.* and Schwarz *et al.*[20,21] The substrate, i.e. CMC (for CMCase activity) at a concentration of 0.1% was incorporated into the separating gel before addition of ammonium persulphate and TEMED for polymerization. After electrophoresis, the gel was sliced in two halves, one half was stained with Coomasie brilliant blue R-250. On the other hand, the second half was used for visualizing the enzymatic activities *in situ*. The second half of the gels was treated with 25% isopropanol for 25-30 min followed by incubation at 50 °C for 25 min. For CMCase activity, 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 stain was totally removed and the background was clear.

Determination of stickies and ink particle size distribution

Micro-stickies were determined in control, chemically deinked and enzymatically (under optimum conditions) deinked pulps with the help of a Pulmac master screen and image analyzer (Paprican Microscanner). The pulps were passed through a Pulmac master screen (customized equipment for collection of micro-stickies), using 0.15 mm slot screen and quantified by using an image analysis. The total content of micro-stickies was calculated (TAPPI T 563 om-03 "Equivalent black area [EBA] and count of visible dirt in pulp, paper and paperboard by image analysis").[22] Residual ink analysis (ink particle size distribution) was determined by Paprican Ink Scanner.[22]

Statistical analysis

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

RESULTS AND DISCUSSION

Isolation and identification of strains AT-2 and AT-3

The fungal strains were isolated by the enrichment culture technique, using moist wheat bran, and incubated at 37 °C. The cellulase production ability of these strains on CMC-agar plate was observed after primary screening based on the clear zone diameter following 0.1% Congo red staining. All the nine isolates hydrolyzed the CMC-agar plates, but AT-2 and AT-3 showed the maximum clear zone diameter. In secondary screening, nine isolates were subjected to produce cellulases using Mandels and Weber's modified medium with rice straw as substrate, under SSF conditions (Table 1). Out of nine, two fungal strains, namely AT-2 and AT-3, produced maximal cellulase activities, i.e. 7.8 and 10.3 IU/mL respectively. These two fungal strains were selected for further studies based on their higher cellulase activities.

Table 1
Enzyme production from the fungal isolates under SSF conditions

Sl. No.	Fungal strain nos	Cellulase activity, IU/mL
1	AT-2	7.8±0.69
2	AT-3	10.3±0.94
3	AT-4	4.12±0.37
4	AT-5	3.70±0.34
5	AT-6	6.50±0.58
6	AT-7	4.25±0.38
7	AT-8	5.73±0.49
8	AT-10	2.0±0.18
9	AT-13	1.3±0.12

± refers standard deviation. Fermentation conditions: rice straw, 5 g, nutrient salt solution, 15 mL, pH 4.8, temperature, 30 °C; incubation period, 5 days

Table 2
Comparison of solid-state and submerged fermentation conditions for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Fermentation	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/ mL	FPase activity, IU/ mL	CMCase activity, IU/mL	FPase activity, IU/ mL
1	SmF	5.1±0.42	0.56±0.05	6.8±0.59	0.72±0.08
2	SSF	8.9±0.81	1.00±0.09	11.35±0.92	1.33±0.20

Fermentation conditions: submerged fermentation: rice straw, 5 g, nutrient salt solution, 15 mL, pH 4.8, temperature, 30 °C, incubation period, 5 days; solid-state fermentation: rice straw, 5 g, nutrient salt solution, 15 mL, pH 5.3, temperature, 35 °C; incubation period, 5 days

The Indian Agricultural Research Institute (IARI), New Delhi (India), identified these two fungal strains AT-2 and AT-3 as *Aspergillus flavus* AT-2 and *Aspergillus niger* AT-3, respectively. Both of these strains were deposited in the Indian Type Culture Collection, Plant Pathology Division, IARI, New Delhi, under ITCC Culture Nos. 6324 and 6325, respectively.

Comparative analysis of different fermentation conditions

Table 2 reveals a comparison between SSF and SmF on cellulase production abilities of both fungal strains, i.e. *A. flavus* AT-2 and *A. niger* AT-3. The CMCase activities of *A. flavus* AT-2 and *A. niger* AT-3 under SSF conditions were of 42.69 and 40.08%, respectively, which were higher than those obtained under SmF conditions. In the same way, FPase activity of *A. flavus* AT-2 and *A. niger* AT-3 increased by 44.00 and 45.86% respectively, in SSF compared to SmF. SSF was a simple technique that was easy to apply on a small scale and it had many advantages compared to the SmF,[23] like simple technology, high volumetric productivity, thus reduced downstream processing costs, low water requirement and high enzyme concentration.[24] SSF was predominantly useful for enzyme production by fungi. Most of the filamentous fungi were reported to perform much better in solid substrate than in liquid cultivation, because they were adapted to growing on solid surfaces. An additional but less investigated advantage of SSF might be the enhanced physiological processes in cell adhesion or biofilm formation.[25]

Optimization of critical parameters for cellulase production

SSF was carried out for optimizing various operating parameters, i.e. incubation period, temperature, pH and moisture contents, and the effect of carbon and nitrogen sources was studied in order to get maximum production of cellulases from *A. flavus* AT-2 and *A. niger* AT-3. Using SSF conditions, critical physicochemical and biological factors were analyzed for deciphering the most favourable conditions for achieving enhanced levels of cellulase production.

Table 3
Optimization of inoculum level and incubation period for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Particulars		*A. flavus* AT-2			*A. niger* AT-3		
		CMCase activity, IU/ mL	FPase activity, IU/mL	Protein concentration, mg/L	CMCase activity, IU/ mL	FPase activity, IU/ mL	Protein concentration, mg/L
Inoculums, %	1	1.6 ±0.13	0.20±0.01	0.04±0.03	2.3 ±0.19	0.24±0.02	0.06±0.04
	2	3.8±0.2.8	0.43±0.03	0.34±0.040	4.0±0.28	0.39±0.04	0.38±0.042
	3	5.7±0.47	0.75±0.06	1.40±0.073	6.6 ±0.51	0.71±0.07	1.46±0.078
	4	8.6±0.73	1.00±0.09	2.82±0.18	9.9±0.87	1.30±0.9	3.00±0.21
	5	8.9±0.56	0.85±0.08	2.86±0.16	10.1±0.78	1.70±0.10	3.06±0.19
	6	3.1±0.29	0.50±0.04	1.77±0.13	6.8±0.43	0.52 ±0.07	2.40±0.16
	7	1.4±0.26	0.37±0.03	0.70±0.10	2.9±0.31	0.19 ±0.03	0.75±0.11
	8	0.9±0.17	0.25±0.03	0.05±0.01	1.1±0.30	0.11 ±0.03	0.09±0.03
Incubation periods, days	2	1.7±0.14	0.20±0.01	0.05±0.03	2.1±0.19	0.27±0.02	0.07±0.004
	3	3.7±0.30	0.43±0.03	0.36±0.040	4.0±0.32	0.46±0.0	0.40±0.042
	4	6.5±0.59	0.75±0.06	1.43±0.073	6.8±0.56	0.75±0.07	1.49±0.078
	5	9.2±0.82	1.00±0.09	2.87±0.18	10.14±0.92	1.18±0.11	3.03±0.21
	6	7.8±0.67	0.85±0.08	2.90±0.16	8.2±0.73	0.98±0.10	3.08±0.19
	7	4.1±0.35	0.50±0.04	1.80±0.13	6.0±0.52	0.68±0.07	2.44±0.16
	8	3.0±0.24	0.37±0.03	1.06±0.10	3.5±0.33	0.40±0.03	1.28±0.11
	9	2.2±0.20	0.25±0.01	0.57±0.06	2.7±0.23	0.33±0.02	0.90±0.07
	10	0.7±0.05	n.d.	n.d	1.1±0.10	0.09±0.005	0.11±0.02

± refers to standard deviation, n.d. = not determined. Fermentation conditions: rice straw, 5 g, nutrient salt solution, 15 mL, pH 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3, respectively, temperature, 30 and 35 °C for *A. flavus* AT-2 and *A. niger* AT-3, respectively, incubation period, 5 days

Optimization of inoculum level for cellulase production

The effect of inoculum doses varying from 1 to 8% on enzyme production by *A. flavus* AT-2 and *A. niger* AT-3 is depicted in Table 3. Table 3 reveals that an inoculum dose of 5% under SSF conditions was optimum. The CMCase and FPase activities of *A. flavus* AT-2 and *A. niger* AT-3 declined beyond an inoculum dose of 5%. The decrease in cellulase production was due to clumping of the cells that could reduce the sugar and oxygen uptake and also the enzyme release.[26] Larger inoculum size was detrimental to the fungal growth and cellulase production, apart from adding to the fermentation cost. Other researchers observed that an inoculum dose varying between 4-6% was optimum for maximum production of cellulase.[26]

Optimization of incubation period for cellulase production

The effect of incubation period on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 is shown in Table 3. The CMCase activities of *A. flavus* AT-2 and *A. niger* AT-3 increased steadily with increasing incubation period and attained maximum (9.20 and 10.14 IU/mL) on the 5th day of incubation, which corresponded to mycelial biomass in terms of protein concentration. The FPase activities on the 5th day of incubation were observed to be of 1.00 and 1.18 IU/mL, respectively, for the strains *A. flavus* AT-2 and *A. niger* AT-3. On longer incubation, enzyme activities of both strains decreased, while protein biomass continued to increase from the 2nd to the 6th day of incubation for both strains and then became nearly constant. It suggests that the enzyme production is dependent on biomass, but only during the exponential phase of fungus growth. As cellulases and xylanases are part of the primary metabolites, they are produced during the exponential phase of growth, and at the onset of the death phase, the enzyme secretion starts decreasing.[27]

Optimization of incubation temperature for cellulase production

Temperature significantly influenced the growth, development and, in general, metabolic activities of an organism. Hence, it was essential to optimize temperature for maximum cellulase production in both fungal strains under SSF, using treated rice straw as carbon source. Table 4 shows that *A. flavus* AT-2 produced maximum CMCase

(9.50 IU/mL) and FPase (1.17 IU/mL) activities at 30 °C, whereas *A. niger* AT-3 produced maximum CMCase (10.75 IU/mL) and FPase (1.30 IU/mL) activities at 35 °C, hence proving their mesophilic nature. The decrease in cellulase production levels might be possibly due to lower transport of substrate across the cells at lower temperature, causing lower yield of the product. At higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of the enzymes of the metabolic pathway, resulting in lower production of the metabolites.

Optimization of medium pH for cellulase production

Among the various operating factors, the pH of the medium was correlated to the enzymatic adsorption, thus significantly affecting the saccharification process.[16] Table 4 reveals that maximal CMCase activities of *A. flavus* AT-2 and *A. niger* AT-3 were of 9.50 and 12.73 IU/mL at pH 4.8 and 5.3, respectively. FPase activities were also found to be maximum (1.4 and 1.73 IU/mL) at the same pH values for both strains *A. flavus* AT-2 and *A. niger* AT-3, respectively. A further increase in pH reduced the CMCase activity of both fungal strains. The reason for decreasing production at higher pH was probably due to proteolytic inactivation of the cellulase. Hence, it suggested that slightly acidic pH values favoured cellulase production, while when further increasing pH, the cellulase activity decreased gradually, which is in agreement with earlier results of other researchers.[28] The H^+ concentration in the fermentation medium thereby had a profound effect on the enzyme production.

The optimal pH for cellulase production from *A. niger* was reported between 6.0 and 7.0 by Akiba *et al*.,[29] while a pH range of 4.5-4.8 was favourable for cellulase production by *Trichoderma reesei* and *Aspergillus phoenicis*.[30] The pH range 4.6-5.0 was found suitable for CMCase, FPase and β-glucosidase production with *Aspergillus ornatus* and *Trichoderma reesei* ATCC-26921.[30]

Optimization of moisture contents for cellulase production

The moisture levels in SSF medium influenced microbial growth and product biosynthesis. Table 4 shows the effect of moisture content on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3. Table 4 reveals that both fungal strains produced maximum cellulase (CMCase) titre (*A. flavus* AT-2, 12.75 IU/mL and *A. niger* AT-3, 16.80 IU/mL), when treated rice straw was moistened with NSS in a ratio of 1:3 and it declined (11.0 and 14.2 IU/mL, respectively) with further increase in substrate to moisture ratio of 1:3.5. Also, a reduction in moisture content (1:2.5) led to depletion in cellulase production in terms of CMCase activity by the strains *A. flavus* AT-2 (11.85 IU/mL) and *A. niger* AT-3 (15.0 IU/mL). It was observed that the moisture enabled better utilization of the substrate by the microorganisms and the efficiency of mass transfer in the solid phase particles depended on the substrate characteristics and the appropriate moisture under SSF growth conditions.[31,32] But further increase in moisture influenced the enzyme production negatively. It reduced the surface area of the particles and made the water film thicker, which affected the accessibility of the air to the particles. The moisture level below optimum led to reduced solubility of the nutrients of the solid substrate and lower degree of substrate swelling.[32]

Effect of carbon sources on cellulase production

Generally, the production of cellulases was shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate was important for high titre of cellulase production.[33] Table 4 depicts that rice straw (untreated) was the most suitable substrate, which induced cellulase (*A. flavus* AT-2, 10.14 IU/mL and *A. niger* AT-3, 14.22 IU/mL) and FPase (*A. flavus* AT-2, 0.90 IU/mL and *A. niger* AT-3, 1.12 IU/mL) productions by both strains up to the highest levels, followed by untreated wheat bran, wheat straw, sugarcane bagasse and rice bran. The CMCase (*A. flavus* AT-2, 13.00 IU/mL and *A. niger* AT-3, 17.27 IU/mL) and FPase (*A. flavus* AT-2, 1.40 IU/mL and *A. niger* AT-3, 2.18 IU/mL) production was further improved using treated rice straw as carbon source instead of untreated rice straw. Rice straw was considered to be the best substrate for cellulase induction, as it contained sufficient nutrients and was able to remain loose in moist conditions, thereby providing good aeration and large surface area, which could be used by fungi for growth and metabolic activity. Rice straw contained 32-47% of cellulose and 19-27% of hemicelluloses.

Table 4
Optimization of incubation temperature, initial pH, solid substrate:moisture content, carbon sources, inorganic nitrogen sources, organic nitrogen sources, phosphorus, organic acids, surfactant, soluble sugars for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Particulars	Variation in conditions	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/ mL	CMCase activity, IU/mL	FPase activity, IU/ mL
*Temperature, °C	25	6.57±0.50	0.71±0.05	7.80±0.66	0.87±0.07
	30	9.50±0.84	1.17±0.11	9.50 ±0.82	1.02±0.10
	35	8.30±0.71	1.01±0.09	10.75± 0.94	1.30±0.12
	40	7.80±0.64	0.90±0.08	9.90±0.89	1.13±0.09
	45	7.00±0.58	0.81±0.06	8.2±0.68	0.93±0.08
	50	5.80±0.43	0.50±0.04	6.95±0.56	0.7±0.05
**pH	4.3	6.6±0.52	0.9±0.07	7.2±0.60	0.98± 0.08
	4.8	9.5±0.83	1.4±0.13	10.70±0.88	1.40±0.23
	5.3	9.1±0.80	1.2±0.11	12.73±1.01	1.73±0.25
	5.8	8.2±0.76	1.0±0.09	11.00±0.95	1.34±0.21
	6.3	7.09±0.67	0.9±0.082	9.2±0.78	1.27±0.19
	6.9	5.33±0.40	0.75±0.067	6.7±0.58	0.87±0.16
	7.5	1.75±0.15	0.11±0.01	3.12±0.29	0.50±0.04
	8.0	0.72±0.06	n.d.	1.25±0.12	0.12±0.01
***Solid substrate: moisture content	1:2.0	10.26±0.77	1.42±0.14	13.0±1.02	1.80±0.24
	1:2.5	11.85±0.90	1.57±0.20	15.0±1.17	2.01±0.32
	1:3.0	12.75±0.97	1.91±0.27	16.80±1.22	2.34±0.40
	1:3.5	11.0±0.85	1.46±0.16	14.2±1.12	1.95±0.28
	1:4.0	10.11±0.67	1.2±0.12	12.8±0.99	1.6±0.20
****Carbon sources	$^{\phi}$SB	6.00±0.57	0.53±0.04	8.9±0.71	0.77±0.05
	$^{\phi}$WS	6.80±0.64	0.59±0.05	9.5±0.75	0.86±0.06
	$^{\phi}$RB	3.52±0.45	0.28±0.02	5.0±0.56	0.44±0.03
	$^{\phi}$WB	9.85±0.93	0.81±0.07	12.75±1.10	1.02±0.09
	$^{\phi}$RS	10.14±0.98	0.90±0.08	14.22±1.15	1.12±0.10
	$^{\phi\phi}$RS	13.00± 1.12	1.40±0.16	17.27±1.25	2.18±0.37
	$^{\phi\phi}$RS+WB(1:1)	12.12±1.08	1.28±0.13	15.75±1.17	1.90±0.27
	$^{\phi\phi}$RS+RB (1:1)	8.57±0.87	0.75±0.04	11.39±0.87	0.95±0.07
	$^{\phi\phi}$RS+SB (1:1)	10.20±1.00	0.95±0.07	13.9±1.07	1.08±0.09
	$^{\phi\phi}$RS+WB(1:1)	11.00±1.02	1.10±0.11	15.64±1.15	1.80±0.24
$^{\Phi 1}$Inorganic nitrogen sources	$(NH_4)_2 SO_4$	13.46±1.01	1.49±0.18	17.65±1.35	2.23±0.33
	$(NH_4)_2$ HPO4	12.68±0.95	1.34±0.15	16.11±1.14	2.00±0.30
	KNO_3	10.90±0.82	1.00±0.9	14.00±1.06	1.65±0.16
	$NaNO_3$	10.40±0.75	0.90±0.8	13.43±1.00	1.46±0.13
	$(NH_4) NO_3$	11.50±0.87	1.10±0.11	15.37±1.10	1.92±0.23
$^{\Phi\Phi 1}$Organic nitrogen sources	Yeast extract	13.40±0.98	1.42±0.15	17.10±1.15	1.98±0.26
	Peptone	12.34±0.90	1.24±0.13	15.93±1.10	1.60±0.20
	Urea	13.01±0.95	1.32±0.14	16.24±1.12	1.72±0.22
	Soya bean meal	11.65±0.82	1.18±0.11	14.58±1.03	1.49±0.17
	Beef extract	10.85±0.77	0.97±0.09	13.00±0.91	1.36±0.14
$^{\Phi\Phi\Phi 2}$Phosphorus sources	KH_2PO_4	13.48±0.98	1.10±0.10	17.37±1.19	1.21±0.17
	K_2HPO_4	12.97±0.96	1.00±0.08	16.8±1.14	1.13±0.12
	Sodium β-glycero phosphate	8.3±0.74	0.66±0.05	10.24±0.89	0.80±0.07
	Tetra sodium pyrophosphate	7.1±0.67	0.47±0.03	9.8±0.80	0.71±0.06
$^{\Phi\Phi\Phi\Phi 3}$Organic acids	Acetic acid	13.40±0.95	1.01±0.09	17.30±1.16	1.34±0.20
	Citric acid	10.5±0.83	0.94±0.06	13.9±0.99	1.16±0.14
	Propionic acid	8.0±0.7	0.65±0.05	11.11±0.88	0.87±0.06
	Oxoglutaric acid	7.6±0.62	0.56±0.04	10.9±0.85	0.82±0.05
	Succinic acid	13.5±0.98	0.97±0.07	17.41±1.21	1.39±0.22

ΦΦΦΦΦ3 Surfactants	Tween-20	10.80±0.8	1.11±0.10	13.61±1.00	1.28±0.18
	Tween-40	10.39±0.87	1.00±0.09	13.90±1.17	1.40±0.21
	Tween-60	11.87±0.92	1.24±0.17	14.70±1.23	1.48±0.22
	Tween-80	13.65±0.96	1.46±0.20	17.89±1.40	2.05±0.30
	Triton-x	09.70±0.75	0.93±0.06	12.32±0.91	1.18±0.16
ΦΦΦΦΦΦ4 Soluble sugars	Glucose	14.8±0.98	1.53±0.18	19.2±1.5	2.01±0.26
	Lactose	13.7±0.90	1.40±0.16	18.1±1.4	1.87±0.20
	Cellobiose	17.2±1.25	1.85±0.19	22.93±1.7	2.30±0.31

± refers to standard deviation; [1] 2.1 g/L, [2] 2.0 g/L, [3] 0.1 g/L, [4] 0.2%; SB = sugarcane bagasse, WS = wheat straw, RB = rice bran, WB = wheat bran, RS = rice straw-RS, $^{\phi}$ untreated, $^{\phi\phi}$ pretreated
Fermentation conditions: rice straw, 5 g, nutrient salt solution, 15 mL, pH 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3, respectively, *temperature = varied, **temperature = 30 °C and 35 for *A. flavus* AT-2 and *A. niger* AT-3, respectively; **pH = varied, ***solid substrate:moisture content = varied, ****carbon sources = varied, $^{\Phi}$inorganic nitrogen sources = varied, $^{\Phi\Phi}$organic nitrogen sources = varied, $^{\Phi\Phi\Phi}$phosphorous sources = varied, $^{\Phi\Phi\Phi\Phi}$organic acids = varied, $^{\Phi\Phi\Phi\Phi\Phi}$surfactants = varied, $^{\Phi\Phi\Phi\Phi\Phi\Phi}$soluble sugars = varied

The cellulose and hemicelluloses contents of rice straw could be hydrolyzed chemically or enzymatically. The cell wall of rice straw was made chiefly of cellulose, hemicelluloses and lignin. The cellulose and hemicelluloses were rather easily attacked by cellulolytic organisms. However, it was the lignin-cellulose complex (LCC) that made straw difficult to digest. Digestibility of lignocellulosic material was generally inversely correlated to the amount of lignin present in the substrates.[34]

Effect of nitrogen sources on cellulase production

Table 4 shows the effect of different inorganic nitrogen sources on cellulase production by both strains. The maximum CMCase (*A. flavus* AT-2, 13.46 IU/mL and *A. niger* AT-3, 17.65 IU/mL) and FPase (*A. flavus* AT-2, 1.49 IU/mL and *A. niger* AT-3, 2.23 IU/mL) activities were observed with $(NH_4)_2SO_4$. The second highest cellulase (*A. flavus* AT-2, 12.68 IU/mL and *A. niger* AT-3, 16.11 IU/mL) and FPase (*A. flavus* AT-2, 1.34 IU/mL and *A. niger* AT-3, 2.00 IU/mL) activities were observed with $(NH_4)_2HPO_4$. Gokhle *et al.* observed that $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were the best inorganic nitrogen sources for the production of CMCase, β-glucosidase and xylanase activity by *A. niger* NCIM1207 strain.[35]

Table 4 shows the effect of different organic nitrogen sources on cellulase production. The maximum CMCase (*A. flavus* AT-2, 13.40 IU/mL and *A. niger* AT-3, 17.10 5 IU/mL) and FPase (*A. flavus* AT-2, 1.42 IU/mL and *A. niger* AT-3, 1.98 IU/mL) activities were observed with yeast extract. Experiments regarding the effect of various organic nitrogen sources on cellulase production demonstrated that there was a substantial increase in the enzyme activity when the medium was supplemented with complex nitrogen sources like yeast extract and urea. These results are in agreement with a previous report by Gao *et al.*[36] whereas yeast extract was found to be the best organic nitrogen source for enhancing endoglucanase production compared to peptone and urea. The favourable effect of yeast extract on cellulase production by white-rot fungi was also observed by Johansson.[37]

Effect of phosphorous sources on cellulase production

Table 4 shows the effect of different phosphorus sources on cellulase production. The maximum CMCase (*A. flavus* AT-2, 13.48 IU/mL and *A. niger* AT-3, 17.37 IU/mL) and FPase (*A. flavus* AT-2, 1.1 IU/mL and *A. niger* AT-3, 1.21 IU/mL) activities were observed with potassium dihydrogen phosphate. Garg and Neelkantan also demonstrated potassium dihydrogen phosphate to have an inductive effect on cellulase activity.[38]

Effect of organic acids

Table 4 shows the effect of different organic acids (0.1%) on the production of cellulase. The maximum CMCase (*A. flavus* AT-2, 13.50 IU/mL and *A. niger* AT-3, 17.41 IU/mL) and FPase (*A. flavus* AT-2, 0.97 IU/mL and *A. niger* AT-3, 1.39 IU/mL) activities were observed with succinic acid. The CMCase activity induced by acetic acid in the medium resembled that of succinic acid. Singh *et al.* observed the increased production of CMCase, FPase and β-glucosidase with acetic acid.[39] The pH of the system during cellulolytic activity increased slightly during incubation and might be regulated by addition of organic acids.

Effect of surfactants on cellulase production

The effect of various surfactants (0.1%, *v*/*v*) on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 is shown in Table 4. The maximum CMCase (*A. flavus* AT-2, 13.65 IU/mL and *A. niger* AT-3, 17.89 IU/mL) and FPase (*A. flavus* AT-2, 1.46 IU/mL and *A. niger* AT-3, 2.05 IU/mL) activities were observed with surfactant Tween-80. According to a study carried out by Dominguez *et al*.,[40] Tween-80 influenced the morphology of *Trichoderma reesei Rut* C-30, as well as the enzyme production. The emulsification with Tween-80 led to higher cellulase activities, presumably by causing increased permeability of the cell membranes and/or by promoting the release of cell-bound enzymes.

Effect of soluble sugars concentration on cellulase production

Table 4 shows the effect of soluble sugars, like glucose, lactose and cellobiose, on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3. The addition of 0.2% cellobiose to the medium increased CMCase activities by 17.2 and 22.93 IU/mL, respectively, for *A. flavus* AT-2 and *A. niger* AT-3, while FPase activities by 1.85 and 2.30 IU/mL, respectively. The CMCase activity with glucose and lactose was minimum in comparison with cellobiose. The presence of soluble sugars like glucose/cellobiose or any other soluble sugars along with cellulose might have initially led to cell growth, which was then induced by cellulose for enzyme production. Waki *et al.* proposed a mechanism of cellulase biosynthesis and stated that cellulose degraded into soluble oligosaccharides and cellobiose.[41] Cellobiose then entered the cell wall and degraded into glucose due to cellobiase (β-glucosidase). The glucose acted as catabolite repressor and thus cellobiose acted as inducer.[41] Cellobiose might act as an effective inducer of cellulase in fungi and of aryl-β-glucosidase in *Neurospora*. Shiang *et al.* proposed a possible regulatory mechanism of cellulase biosynthesis. According to them, sugar analogues, i.e. cellobiose, glucose, sucrose, sorbose, xylose etc., at a particular concentration, induced cellulase activator molecule (CAM), a cellulase regulatory protein.[42] CAM might or might not be the same component as suggested by Stutzenberger, who described the regulation of cellulase synthesis using a modification of arabinose.[43] The formation rate and yield of CAM was reported to be dependent on substrates, substrate concentration and some unknown factors imparted by moderators. Several investigations have so far indicated that cellulases are inducible enzymes. An increased rate of endoglucanase biosynthesis in *Bacillus* species has been reported in the presence of cellobiose or glucose (0.2%) added to the culture medium.

Biochemical characterization of cellulase

Effect of pH on the activity

The robustness of the cellulolytic enzymes is the key factor for industrial applications. Table 5 shows the effect of pH on the cellulase activity of *A. flavus* AT-2 and *A. niger* AT-3 at 50 °C for 30 min of reaction time. Cellulases produced by *A. flavus* AT-2 and *A. niger* AT-3 were active in the pH range of 4.0 to 7.5 and maximum cellulase activity was obtained at pH 5.0 for *A. flavus* AT-2 (17.31 IU/mL) and at pH 5.5 for *A. niger* AT-3 (24.50 IU/mL). Cellulase activities of *A. flavus* AT-2 and *A. niger* AT-3 started decreasing above buffer pH 5.0 and 5.5, respectively. It was observed that at neutral pH (7.0), *A. flavus* AT-2 retained about 54% of its optimum cellulase activity, while *A. niger* AT-3 retained only 65% of its optimum cellulase activity. At pH 4.0, *A. flavus* AT-2 retained 84% and *A. niger* AT-3 retained 90% of its activity compared to optimum.

Paul *et al*. earlier reported in their study that the optimum pH for the enzyme produced by *A. niger* was pH 3.8-4.0 and it was stable at 25 °C over the range of pH 1-9; the maximum activity (at pH 4.0) was obtained at 45 °C, while cellulase was more stable to heat treatment at pH 8.0 than at pH 4.0.[44] They also found that kinetic studies gave pK values between 4.2 and 5.3 for groups involved in the enzyme-substrate complex. Similar results were obtained by Aboul-Enein and his coworkers in the case of thermo active cellulase produced from thermophilic actinomycete.[45] The enzyme was optimally active at 60 °C and pH 8 and was stable from pH range of 6 to 9, retaining more than 80% of its activity after incubation at room temperature for 12 h.[45]

Effect of temperature on the activity and stability of cellulase

Table 5 shows the effect of temperature on the activity of crude cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3 at their optimum pH, i.e. 5.0 and 5.5 respectively, and reaction time of 30 min. Table 5 reveals that the optimum temperature for the crude cellulase activities of

both fungal strains (*A. flavus* AT-2, 17.44 IU/mL and *A. niger* AT-3, 24.73 IU/mL) was of 50 °C and beyond that cellulase activities were found to decrease. At the temperature of 60 °C, cellulase from *A. flavus* AT-2 retained about 51% of its activity, while that of *A. niger* AT-3 exhibited about 64% of its activity, compared to that at optimum temperature, i.e. 50 °C. When assayed at higher temperatures (70 and 80 °C), both strains lost a large amount of their cellulase activities: *A. flavus* AT-2 could maintain 19 and 4%, while *A. niger* AT-3 retained 29 and 7% of its cellulase activities respectively, as compared to the optimum cellulase activity at 50 °C. *A. niger* AT-3 was found to be slightly more thermo-tolerant, as it showed more tolerance to higher temperature as compared to *A. flavus* AT-2. The thermal stability of enzymes appeared to be a property acquired by a protein through a combination of many small structural modifications that were achieved with the exchange of some amino acids. The variation of canonical forces, e.g. hydrogen bonds, ion-pair interactions and hydrophobic interactions, provided thermozymes resistance at high temperature. The optimal temperature for the crude cellulase activity of both the strains was 50 °C. These results were validated by the finding that the optimum temperature for assaying cellulase activities was generally within the range of 50-65 °C for a variety of microbial strains, e.g. *Thielavia terrestris*-255B, *Myceliophthora fergussi*-246C, *Aspergillus wentii*, *Penicillum rubrum*, *Aspergillus niger* and *Aspergillus ornatus.*[17,39,45]

Table 5
Effect of pH and temperature on cellulase activity of *A. flavus* AT-2 and *A. niger* AT-3

Particulars		*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/ mL	Relative cellulase activity, %	CMCase activity, IU/ mL	Relative cellulase activity, %
pH	4.0	14.56	84	22.03	90
	4.5	16.81	98	22.92	94
	5.0	17.31±1.1	100.00	23.78	97
	5.5	14.29	83	24.50±1.1	100
	6.0	13.27	77	23.35	95
	6.5	12.33	71	21.23	87
	7.0	9.31	54	15.89	65
	7.5	4.98	29	9.19	38
Temperature, °C	40	7.80	60	9.90	63
	50	17.44±0.81	100	24.73±0.81	100
	60	8.92	51	15.72	64
	70	3.36	19	7.13	29
	80	0.73	4	1.62	7

± refers to standard deviation. Assay conditions: pH of buffer 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively, substrate concentration, 20 mg/mL citrate buffer for cellulase activity

Table 6
Reduction in stickies in different deinking processes of sorted office paper

Particulars	Stickies, no/kg	Stickies removal, %
Control	3060	–
Chemical deinking	1774	42.02
Enzymatic deinking		
Cellulase	1652	46.01
Cellulase + Xylanase	1650	46.07
Cellulase + Xylanase + Amylase	1645	46.24
Cellulase +Xylanase + Amylase +Lipase	1573	48.59

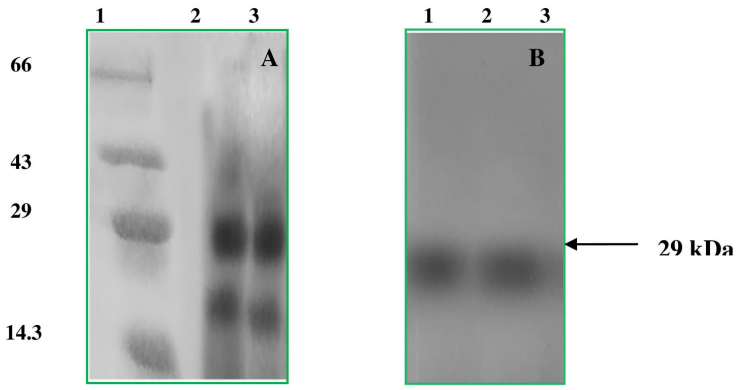

Figure 1: SDS-PAGE (A) and Zymogram (B) analyses of cellulase produced by *A. niger* AT-3

Molecular characterization by SDS PAGE analysis and cellulase activity detection by zymogram analysis

The extra cellular protein profile produced by *A. niger* AT-3, using concentrated crude culture filtrate and CMC as substrate, were analyzed by electrophoresis on SDS-polyacrylamide gel. Molecular weight analysis of the respective bands indicated that cellulase had a molecular weight of 29 kDa (Fig. 1). A wide range of proteins from 10-190 kDa was observed. The variation in the molecular weight of the protein among cellulase components was due to the variation in the conserved region and glycosylation.[46] The bands of molecular masses equivalent to 91.2 kDa, 68 kDa and 52.4 kDa were detected in the native gel, corresponding to the CMCase, FPase and β-glucosidase activities respectively.

Micro-stickies removal in different deinking processes

Stickies originate from adhesives, ink binders and coating binders. Stickies able to pass through a sieve of 100 or 150 μm mesh size (depending on standard) are called micro-stickies, whereas the particles retained on the screen are called macro-stickies. Dissolved and colloidal stickies are called secondary stickies. Stickies are tacky, hydrophobic, pliable organic material found in recycled paper systems. Stickies were composed of a variety of materials, including adhesives, styrene-butadiene latex, rubber, polyvinyl acetate and hot melts etc. They affect both the process efficiency (breaks, formation of deposits on various equipments of the paper machine, reduction of the drying section efficiency due to felts clogging, etc.), and the quality of the final product (presence of spots, holes and other defects).[47] Flotation is very effective in stickies removal, but various enzyme applications were also found effective to remove stickies from waste paper. Chemicals, cellulase, cellulase + xylanase, cellulose + xylanase+amylase and cellulase + xylanase + amylase + lipase deinking processes removed micro-stickies by 42.02, 46.01, 46.07, 46.24 and 48.59%, respectively, compared to control (3060 no/kg) (Table 6). Cellulase alone improved the stickies removal by 4% as compared to control. Further, xylanase and amylase introduced in the mixture did not bring a significant improvement (0.06 and 0.23%, respectively) in stickies removal. The addition of lipase was also significant for improving the stickies removal. It means that xylanase with cellulase acted synergistically for ink removal and amylase effectively degraded starch size and released ink particles from the fiber surface instead of removing stickies. Cellulases and different forms of amylases and xylanases were also found to work as surface cleaning agens during deinking. Lipase attacked the ester constituents of the tonners and sizing material in the paper furnish and released the ink particles from the fiber. As a possibility, the cellulase mixtures could have released sticky particles from fiber surfaces by the same mechanism as used to describe enzymatic toner ink removal.[48] Lipase might work on synthetic stickies substrate in a very different way when stickies become intermingled with printing inks and papermaking additives. Park *et al.* observed reduction in stickies by using cellulase in deinking of mixed office waste and old news print.[49]

Ink particle size distribution in different deinking processes

Proper ink particle size and air bubble ratio was important for good flotation. Ink particle

sizes (specks) in terms of speck numbers and specks/cm^2 were determined in pulps after chemical, enzymatic and chemi-enzymatic deinking processes and were found to range between 8-20, 20-80, 80-224 and 8-2000 µm, respectively. The average speck diameters in the above particle size ranges for the chemical, cellulase, cellulase + xylanase, cellulase + xylanase + amylase and cellulose + xylanase + amylase + lipase, chemicals (100%) + cellulase, chemicals (50%) + cellulose, chemicals (25%) + cellulase deinking processes were found to be, with standard deviations, 12.34±0.46, 35.62±1.32, 122.19±3.6 and 44.64±1.52. Ink particle size in terms of number of specks and number of specks/cm^3 in chemical and enzymatic deinking processes reduced from top downward (Table 7) in the following order: chemicals > cellulase > cellulase + xylanase > cellulase + xylanase + amylase > cellulase + xylanase + amylase + lipase. It means that the mixture of enzymes was capable of removing all sizes of ink particles from the deinked pulp.

Table 7
Ink particle size distribution in different deinking processes of sorted office paper

Particulars	Ink particle size range, µm			
	8-20	20-80	80-224	8-2000
	Conventional deinking			
Chemical deinking				
Number of specks	411	303	33	347
Number of specks/cm^2	1409	1039	113	1190
Average speck diameter, µm	12.96	38.26	124.28	47.29
	Enzymatic deinking			
Cellulase				
No. of specks	383(-6.81)	243(-19.14)	31(-6.06)	270(-22.19)
No. of specks/cm^2	1313(-6.81)	833(-19.82)	106(-6.19)	926(22.18)
Average speck diameter (µm)	12.65	36.08	123.67	45.73
Cellulase + xylanase				
No. of specks	366(-10.94)	248(-18.15)	28(-15.15)	276(-20.46)
No. of specks/cm^2	1255(-10.92)	850(-18.19)	95(-15.92)	947(-20.42)
Average speck diameter (µm)	12.60	35.29	119.81	44.42
Cellulase + xylanase + amylase				
No. of specks	331(-19.46)	201(-33.66)	24(-27.27)	238(-31.41)
No. of specks/cm^2	1135(-19.44)	689(-33.68)	83(-27.43)	816(-31.42)
Average speck diameter (µm)	12.17	35.25	119.47	43.26
Cellulase + xylanase + amylase + lipase				
No. of specks	182(-55.71)	190(-37.29)	19(-42.42)	178(-48.70)
No. of specks/cm^2	608(-56.84)	644(-38.01)	65 (-42.47)	607(-48.99)
Average speck diameter (µm)	12.01	34.44	116.45	42.94
	Chemi-enzymatic deinking			
(C100%+E)				
No. of specks	111(-72.99)	67(-77.88)	7(-78.87)	93(-73.19)
No. of specks/cm^2	377(-73.24)	224((-78.44	23(-79.64)	319(-73.19)
Average speck diameter (µm)	12.16	35.29	125.87	44.15
(C50%+E)				
No. of specks	146(-64.47)	85(-71.94)	12(-63.63)	113(-67.43)
No. of specks/cm^2	500(-64.51)	291(-71.99)	41(-63.71)	377(-68.31)
Average speck diameter (µm)	12.11	35.26	124.28	44.26
(C25%+E)				
No. of specks	190(-53.77)	127(-58.08)	19(-42.42)	145(-58.21)
No. of specks/cm^2	656(-53.58)	435(-58.13)	65(-42.47)	486(-59.15)
Average speck diameter (µm)	12.09	35.12	123.72	45.11
Average	12.34±0.46	35.62±1.32	122.19±3.6	44.64±1.52

- sign shows % reduction compared to chemical deinking, ± shows standard deviation

The number of specks in cellulose + xylanase + amylase + lipase deinked pulp was reduced by 55.71% in 8-20 µm size range, 37.29% in 20-80 µm size range, 42.42% in 80-224 µm size range and 48.70% in 8-2000 µm size range, compared to chemically deinked pulp. Similarly, the number of specks/cm^3 was reduced by 56.84% in 8-20 µm size range, 38.01% in 20-80 µm size range, 42.47% in 80-224 µm size range and 48.99% in 8-2000 µm size range, compared to chemically deinked pulp (Table 7). Regardless of ink type or printing process, the enzymatic treatment was also helpful in reducing ink particle size. On the contrary, in chemi-enzymatic deinking processes, ink particle sizes observed in the ranges of 8-20, 20-80 and 80-224 µm increased in the following order: chemicals (100%) + enzyme < chemicals (50%) + enzyme < chemicals (25%) + enzyme. Whereas, the ink particle sizes observed in the range of 8-2000 µm show a decreasing trend for the above mentioned chemi-deinking processes (Table 7). Smaller ink particles had a greater tendency to redeposit onto the fibers than larger ones.

CONCLUSION

The optimum CMCase (17.24 IU/mL), FPase (1.92 IU/mL), β-glucosidase (0.69 IU/mL) and xylanase activities (5.73 IU/mL) and fungal protein concentration (2.90 mg/L) for *A. flavus* AT-2, and CMCase (24.32 IU/mL), FPase (2.47 IU/mL), β-glucosidase (0.82 IU/mL) and xylanase activities (4.80 IU/mL) and protein concentration (3.07 mg/L) for *A. niger* AT-3 were observed under optimum SSF conditions. *A. flavus* AT-2 and *A. niger* AT-3 were active in the pH range of 4.0 to 7.5 and maximum cellulase activity was obtained at pH 5.0 for *A. flavus* AT-2 (17.31 IU/mL) and at pH 5.5 for *A. niger* AT-3 (24.50 IU/mL). The optimum temperature for the crude cellulase activities of both fungal strains (*A. flavus* AT-2, IU/mL and *A. niger* AT-3, IU/mL) was 50 °C, and beyond it cellulase activities were found to decrease. SDS PAGE and zymogram analyses indicated that the molecular weight of cellulase produced by *A. niger* AT-3 was 29 kDa.

Cellulase alone improved the stickies removal by 4%, as compared to the control. Further, xylanase and amylase introduced in the mixture did not bring a significant improvement (0.06 and 0.23%, respectively) in stickies removal. The addition of lipase significantly improved the stickies removal. Ink particle size, in terms of number of specks and number of specks/cm^3, in chemical and enzymatic deinking processes reduced in the following order: chemicals > cellulase > cellulase + xylanase > cellulase + xylanase + amylanase > cellulase + xylanase + amylase + lipase.

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