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Screening of Fungal Resources for the Production of Cellulases and Xylanases

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Authors' contributions

This work was carried out in collaboration among all authors. Author AK designed the study, wrote the protocols and performed experimental work. Author AG managed literature searches and wrote the first draft of the manuscript. Author DD analyzed the results and revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The final screening of fungal isolates aimed at applications based tertiary screening i.e. deinking of mixed office waste paper and saccharification of pearl millet stover and cellulases from selected fungal isolates were characterized.

Study Design: An experimental study.

Methodology: Samples from soil, compost and decaying wood were collected from different habitats and were screened based on growth over CMC-agar medium (primary screening), zone ratios and enzyme activities (secondary screening) and applications such as bio-deinking of mixed office paper and saccharification of pearl millet stover (tertiary screening).

Results: 134 fungal isolates were selected during primary screening based on their growth. In secondary screening, fungal strains showing zone ratio of 3.0 or more were selected for application based tertiary screening. Two fungal isolates AKB-24 and AKB-25 were selected based on their applications in deinking of mixed office waste and saccharification of pearl millet stover after tertiary

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screening. Fungal isolates AKB-25 and AKB-24 were identified as *Aspergillus nidulans* and *Penicillium* sp. Optimum pH for FPase, endoglucanase, and glucosidase activities were 5.0 for both the fungal strains. Cellulases from *A. nidulans* AKB-25 were found moderately thermo-stable with optimum endoglucanase activity at 65ºC and optimal FPase and β-glucosidase activities at 60ºC. The maximal endoglucanase, FPase and β-glucosidase activities were observed at 55ºC for fungal strain *Penicillium sp.* AKB-24. Cellulases from both fungal strains were found stable up to 48 h at 50ºC.

Conclusion: *Aspergillus nidulans* AKB-25 and *Penicillium sp.* AKB-24 were selected based on an extensive screening and enzymes from both fungal strains were found effective in bio-deinking of mixed office waste paper. Enzyme from *Aspergillus nidulans* AKB-25 was also found effective in saccharification of pearl millet stover.

Keywords: Screening; bio-deinking; cellulases; xylanases; Aspergillus nidulans; Penicillium.

1. INTRODUCTION

The pulp and paper industry heavily exploits natural resources like wood, water and energy. Every metric tonne of recycled fibre saves an average of 17 trees, at least 30,000 litres of water, 3000-4000 kWh of electricity and 95% of air pollution [1,2]. The primary objective of waste paper recycling is the removal of printing inks and other contaminants while retaining the optical and strength properties of fibres [3]. Various methods are available for waste paper deinking like heat decolourization [4], irradiation [5], organic solvent-based deinking [6], ultrasound [7], magnetic deinking [8] and chemical deinking [9]. Most of the conventional chemical deinking techniques require large amount of chemical agents [9], resulting in a costly wastewater treatment to meet the environmental regulations [10] and generate large quantity of sludge and effluents with high oxygen demanding material [11] and rest technologies are under experimental conditions.

Alternatively, use of enzyme for detaching the toner particles from fibres has been reported as efficient process and less polluting solution to overcome this disposal problem [12-14]. In most cases, cellulases represent the best choice; however other enzymes hemicellulases, amylases and lipases can help in order to optimize the process depending on the type of paper and ink [3]. Dutt et al. [15] showed in their studies that concoctions of enzymes containing cellulase, xylanase and amylase have been found very effective in deinking of sorted office paper. Fungi producing cocktail of enzymes including cellulase, xylanase and amylase may be a better option for bio-deinking of waste paper.

Although, different commercial efficient cellulases and xylanases from Novozymes and Genencor etc are available for cellulosic biomass hydrolysis but bioethanol production technologies and enzymatic deinking of waste paper require continuous supply of enzymes which may be achieved by on-site/in-house production of cellulases and xylanases. This indigenous enzyme production technology with hydrolysis efficiencies and deinking efficiencies comparable to commercial enzyme preparations can be used for bioethanol production and bio-deinking of waste paper [16]. Cellulases and xylanases are produced by several microorganisms such as bacteria, actinomycetes and fungi, but the fungi are of great interest because they excrete extracellular enzymes in higher quantity [17,18]. Fungal diversity is major resource for lignocellulosics hydrolyzing enzymes like cellulases, xylanases, pectinases and laccases. In the present study, an extensive screening was carried out based on higher enzyme activities, deinking potential and hydrolysis capability of crude enzymes from fungal resources. Use of crude enzyme for enzymatic deinking is economical compared to commercial enzymes which are available in purified form. But, some of fungi have been found to produce pigments along the enzymes and on applying the crude enzyme from such sources may hinder the improvement in brightness of deinked paper [19]. Therefore, it is necessary to screen such microbial sources which can produce enzymes in higher quantity without pigments.

With concept, present study aims at screening of selected fungal isolates based on clear zone diameter, enzyme activities, deinking efficiency and production of reducing sugars after hydrolysis of pearl millet stover and partial characterization of cellulases from *Aspergillus nidulans* AKB-25 and *Penicillium* sp. AKB-24.

2. MATERIALS AND METHODS

2.1 Isolation and Primary Screening

Different decomposing manures, dead and decaying wood and soil enriched with lignocelluloses samples were collected from different sites of Uttar Pradesh, Uttarakhand and Rajasthan using sterilized spatula and polythene bags. One gram of each sample was homogenized manually and transferred to test tube containing sterilized distilled water. It was shaken vigorously and the suspension was subjected to serial dilution. After serial dilution, plating was carried out from appropriate dilutions. Primary screening for cellulase producing fungi was carried out on medium composed of various constituents expressed as g/l: 1.0 carboxymethyl cellulose (CMC), 1.0 peptone and yeast extract, 0.5 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.005 $FeSO_4 \cdot 7H_2O$, 0.0016 $MnSO_4.2H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.002 $CoCl_2.6H_2O$, and 15 agar. Petri-plates were incubated at 30ºC for seven days and selected colonies were isolated, purified on potato dextrose agar (PDA) and maintained over PDA slants at 4ºC.

2.2 Secondary Screening

2.2.1 Screening based on zone ratio and cellulase activity of crude extract

Fungal isolates, selected during primary screening were grown under solid-state fermentation (SSF) using wheat bran as the substrate. Wheat bran (5 g) was moistened with Mandel Weber medium (77.5% initial moisture content) with following composition expressed as g/l: 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 $CaCl_2$, 0.3 $MgSO_4.7H_2O$, 0.1 Tween-80 and trace elements: 0.005 $FeSO_4.7H_2O$, 0.0016 $MnSO_4.7H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.002 $CoCl_2.6H_2O$. The initial pH of Mandel Weber medium for enzyme production was adjusted to 5.5 with 1N HCl or 1N NaOH. Flasks were inoculated with 10^6 spores/gds or five discs of actively growing fungi for spores producing and spores lacking fungi respectively and incubated at 30ºC for 6 days. The enzyme extraction was carried out by addition 50 ml of distilled water to fermented wheat bran before shaking at 150 rpm and 30ºC for 60 min in an incubator shaker. Clear zone assays were performed using 20 µl of enzyme extract on CMC-agar plates which were flooded with an aqueous solution of 0.5% Congo red for 15 min and destained with 1 M NaCl to enhance the visibility [20]. Zone ratio was calculated by dividing clear zone diameter by well diameter. Zone ratio was calculated for crude enzymes extracted from different fungal isolates and fungal isolates showing zone ratio more than 3.0 were selected for further screening.

2.2.2 Deinkability based screening

Deinking experiments were carried out with crude enzyme from different fungal strains which were selected in previous step to assess their ink detachment capabilities on mixed office waste (MOW). MOW paper was collected from Century Paper Mills, Lalkuan, District Nainital (Uttarakhand) India. MOW paper was torn manually into small pieces of size 1.5-2.5 cm^2 and soaked in water at 30ºC for 60 min. Pulping was carried out in a hydrapulper (Universal Engineering Corporation, Saharanpur, India) at 600 rpm for a pulping time of 20 min. After hydrapulping, enzymatic treatment was given at 50ºC for 60 min. The enzyme doses were given on the basis of endoglucanase activity (2 IU/g oven dried pulp basis). Further, enzymatically treated pulp was subjected to ink flotation for 10 min and washing stages (1% consistency) respectively to remove detached ink particles from the pulp. The deinked pulp pads were prepared on Büchner funnel (Tappi T 218 sp-02 "Forming handsheets for reflectance testing of pulp, Büchner funnel procedure") and tested for pulp brightness (Tappi T 452 om-08 "Brightness of pulp, paper, and paperboard, directional reflectance at 457 nm"). Deinking efficiency was calculated as using following formula [15]:

$$\text{Deinking efficiency (\%)} = \frac{B_F - B_P}{B_B - B_P} \times 100$$

Where,

B_P = Brightness after pulping, % (ISO)
B_F = Brightness after flotation, % (ISO)
B_B = Brightness of the sample paper without the presence of ink particles (blank), % (ISO)

Reducing sugars in pulp filtrate after enzymatic deinking were estimated by using DNS method [21].

2.2.3 Screening based on hydrolysis capability of crude enzyme

Hydrolysis experiments were also conducted with fungal strains selected previously based on zone ratio. Hydrolysis capability of crude enzyme was measured in terms of release of reducing sugars. Pearl millet stover of particle size ranging from

250 to 1400 µm after hydrothermal pre-treatment at 180ºC for 15 min was used as substrate for hydrolysis studies. Pretreated pearl millet stover was washed with tap water and directly used for enzymatic hydrolysis without drying. The hydrolysis experiments were carried out maintaining the consistency of treated pearl millet stover at 1% (w/v) and pH 5.0 using crude enzyme from different fungal sources at an enzyme dose of 5 FPU/g of pretreated millet stover. The hydrolysis of substrate was performed at 50ºC for 96 h and shaken at 120 rpm in an incubator shaker (New Brunswick Scientific, Innova® 43, USA). In all the experiments, 0.01% sodium azide was used to prevent the microbial growth during hydrolysis. Hydrolysate samples were centrifuged at 7200 xg for 15 min to remove solids and analyzed for reducing sugars as per Miller method [21].

2.3 Characterization of Cellulases from Fungal Strains from AKB-24 and AKB-25

2.3.1 Optimum pH and temperature

Optimum pH for cellulases from fungal strains AKB-24 and AKB-25 was determined by assaying the cellulases using different reaction buffers of 50 mM i.e. sodium citrate for a pH range of 3.0 to 6.0, sodium phosphate buffer for a pH range of 6.5 to 8.0 and glycine-NaOH buffer for a pH of 9.0 at a fixed temperature (50ºC). Effect of temperature on enzyme activity was also studied by incubating the reaction mixture at different reaction temperatures varying from 40 to 75ºC with a gap of 5ºC using sodium citrate buffer of 50 mM for maintaining a pH of 5.0 for both of the fungal strains.

2.3.2 Thermo-stability and pH stability

Thermal and pH stabilities of cellulases were determined by incubating the enzymes at different holding times i.e. 1, 3, 6, 24 and 48 h, pH 5.0 (optimum pH) and at two different temperatures i.e. 60ºC and 50ºC for the fungal strain AKB-25. Likewise, enzyme from fungal strain AKB-24 was incubated at the same conditions except temperature i.e. 50ºC and 55ºC respectively.

2.3.3 Partial purification of cellulases by ammonium sulphate precipitation

The cell free supernatant of crude enzyme was subjected to fractional ammonium sulphate precipitation. 200 mL of crude enzyme was taken and finely powdered AR grade ammonium sulphate at different saturation levels of (10% to 100 % w/v) was added slowly to crude enzyme at 4ºC with continuous stirring. Incubating the samples overnight at 4ºC, precipitate was obtained after centrifugation at 9,000 xg for 30 min. Precipitate was dissolved with minimal amount of citrate buffer (0.05 M; pH 5.5) and each fraction was tested for protein and endoglucanase activities. The fractions which showed significantly high activity were pooled and dialyzed to remove ammonium sulphate from enzyme solution. Enzyme salt solution was dialyzed against the same buffer with dialysis membrane at 6ºC for 24 h. Buffer was changed frequently during dialysis till no more ammonium sulphate detected. The undissolved dialysate was removed by centrifuging at 9,000 xg for 20 min at 4ºC.

2.3.4 Effect of cations on partially purified cellulases

Effect of cations on endoglucanase activity was studied by incubating the reaction mixture with various cations (Na^{+}, K^{+}, Al^{+++}, Ca^{++}, Cd^{++}, Co^{++}, Cu^{++}, Fe^{++}, Hg^{++}, Mn^{++}, Mg^{++}, Ni^{++}, Pb^{++}, and Zn^{++}) at two different concentrations (1 and 10 mM) at 50ºC and pH 5. Enzyme-cation mixture was incubated at room temperature for 1 h before it was used in cellulase assay. The residual activities were measured in presence of salt of each ion. Enzyme activity in absence of cations was taken as 100%.

2.4 Enzyme Assays

Endoglucanase (CMCase) and filter paper activity (FPase) activities were estimated by incubating the crude enzyme with carboxymethyl cellulose (2% w/v, medium viscosity) and Whatman filter paper-1 of dimensions 1×6 cm (50 mg) according to Ghose method [22]. Likewise, β-glucosidase, xylanase and amylase activities were estimated by incubating the crude enzyme p-nitrophenyl-β-D-glucopyranoside (5mM), beech wood xylan (1% w/v) and starch (1% w/v) respectively by dissolving in citrate buffer (50 mM) at 50ºC and pH 5.5 according to Bailey et al. [23] and Wood and Bhat [24] methods.

3. RESULTS AND DISCUSSION

3.1 Screening of Fungal Isolates

The basis of primary screening was the growth of fungal isolates on CMC-agar medium and 134

fungal isolates were selected for secondary screening. Enzymes produced by each fungal isolate on wheat bran as the substrate under SSF conditions were subjected to zone assay using 20 µl of crude extract from each isolate (Fig. 1). In secondary screening, fungal strains showing zone ratio of 3.0 or more were selected to observe their potential for deinking and bio-conversion of biomass (Table 1). Some fungi may produce pigments and their presence in crude enzyme preparations may affect pulp brightness adversely [19].

Molecular size and enzyme structure also determine ability of enzymes to access substrate through the fibre matrices [12]. It is generally accepted that three components of cellulases are required to hydrolyse cellulose into glucose monomers are: exo-1,4- β-glucanases, endo-1,4-β-glucanases, and β-glucosidases. Endoglucanases cleave cellulose chains in the middle and creates free chain ends. Exo-1,4- β-glucanases degrade molecules further from by removing cellobiose units from free chain ends and β-glucosidase converts the cellobiose units into glucose molecules. However, some fungi like *Trichoderma reesei* are poor in β-glucosidase, which results accumulation of cellobiose that will cause severe feedback inhibition to the cellulase reaction. Therefore, a cellulase complex system is required for effective hydrolysis of cellulose [25,26].

The choice of an enzyme preparation is more dependent on the characteristics of the substrate rather than on standard enzyme-activities measured [27]. Therefore, the selection of enzymes aimed at applications based tertiary screening i.e. deinking and saccharification. With this background, all the twelve fungal isolates were selected for tertiary screening.

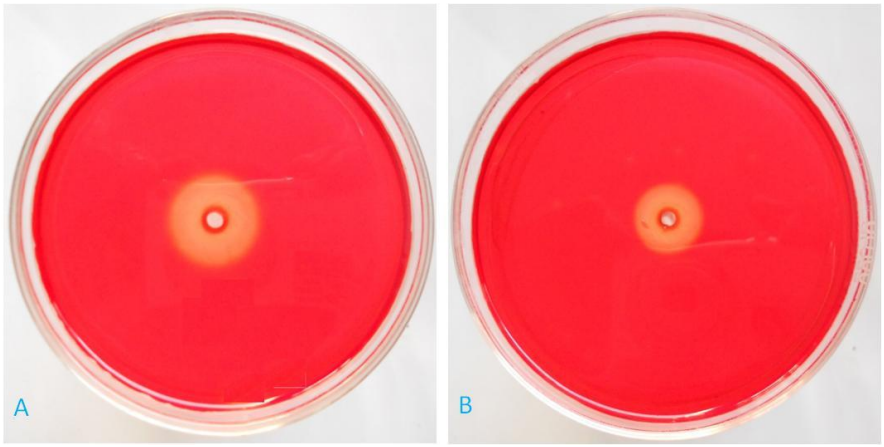

Fig. 1. Zone assay (A) *Penicillium* sp. AKB 24, (B) *Aspergillus nidulans* AKB 25

Table 1. Description of selected fungal isolates

Fungal isolate	Source	Site of isolation	Zone diameter	Zone ratio
AKB-10	Decomposing manure	Muzaffarnagar, Uttar Pradesh	13	3.25
AKB-11	Decomposing manure	Muzaffarnagar, Uttar Pradesh	14	3.50
AKB-12	Dead and decaying wood	Paonta Sahib, Himachal Pradesh	12	3.00
AKB-13	Lignocellulose rich soil	Paonta Sahib, Himachal Pradesh	13	3.25
AKB-14	Dead and decaying wood	Dehradun, Uttarakhand	12	3.00
AKB-15	Dead and decaying wood	Saharanpur, Uttar Pradesh	13	3.25
AKB-16	Lignocellulose rich soil	Jaipur, Rajasthan	14	3.50
AKB-22	Lignocellulose rich soil	Jaipur, Rajasthan	15	3.75
AKB-23	Lignocellulose rich soil	Jaipur, Rajasthan	13	3.25
AKB-24	Lignocellulose rich soil	Jaipur, Rajasthan	17	4.25
AKB-25	Lignocellulose rich soil	Jaipur, Rajasthan	15	3.75
AKB-26	Lignocellulose rich soil	Jaipur, Rajasthan	15	3.75

Zone ratio = Clear zone diameter/well diameter

Different enzyme activities of fungal isolates AKB-14, AKB-15, AKB-23 and AKB-26 were found lower (Table 2). FPase activities of fungal isolates AKB-12, AKB-14, AKB-15, AKB-22, AKB23 and AKB-26 was less than 1.0 FPU/g. Fungal isolates AKB-14, AKB-15, AKB-22, AKB-23 and AKB-24 showed β-glucosidase activities less than 7.0 IU/gds (Table 2). Fungal isolates AKB-13, AKB-15, AKB-23 and AKB-26 produced less than 12.0 IU/gds of endoglucanase activities. Unbleached pulp contains xylan which is important for removing of lignin carbohydrates complexes (LCC) by xylanase during pre-bleaching experiments. Xylanase is also important for saccharification. Among all the fungal isolates, AKB-13, AKB-14, AKB-15, AKB-22, AKB-23 and AKB-26 showed xylanase activities less than 1000 IU/gds) (Table 2). Amylase acts on starch present in the pulp and helps in detaching the ink particle from fibre surface. Starch is added as surface sizing agent as well as dry strength additive during papermaking [15]. AKB-22 and AKB-23 had minimal amylase activity. It is difficult to select a fungal isolate based on only enzyme activities therefore; selection of fungal strains was carried out based on their applications and level of enzyme production.

Among all the fungal isolates, AKB-12, AKB-24 and AKB-25 were found most effective in bio-deinking of MOW paper with deinking efficiency 62.18, 62.18 and 62.73% respectively. Fungal isolates AKB-16, AKB-22, and AKB-25 were found most effective for the hydrolysis of pretreated pearl millet stover and released 254.24, 268.45, and 280.27 mg of reducing sugars per gram of substrate respectively (Table 3). However, fungal isolate AKB-24 was found effective for bio-deinking of MOW as it showed maximum endoglucanase activity (59.20 IU/gds) therefore; it was selected for further studies. Fungal isolate AKB-25 produced 24.0, 1.10 and 27.80 IU/gds of endoglucanase, FPase and β-glucosidase activities respectively and found effective for both deinking of MOW and saccharification of pearl millet stover. Finally, these two fungal strains were selected based on their applications and level of enzyme production for further studies.

3.2 Identification of Selected Fungal Strains

ITS1-5.8S-ITS2 sequencing of the fungal isolates AKB-24 and AKB-25 was carried out at Microbial Culture Collection (MCC), National Centre for Cell Science, Pune (India). On the basis of BLAST search of ITS sequences, fungal strain AKB-24 was identified as *Penicillium* sp. at MCC, National Centre for Cell Science, Pune (India) and submitted with accession number MCC-1031. Fungal isolate AKB-25 was identified as *Aspergillus nidulans* on the basis of morphological characteristics and comparison of ITS rDNA gene sequences, and submitted to NFCCI, Agharkar Research Institute, Pune (India) with accession number NFCCI-2977. The ITS sequences of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 were submitted to GenBank with accession numbers KP734016 and KP734017 respectively.

Table 2. Enzyme activities of selected fungal isolates

Fungal isolates	Endoglucanase (IU/ gds)	FPase (FPU/gds)	β-glucosidase (IU/ gds)	Xylanase (IU/ gds)	Amylase (IU/ gds)
AKB-10	27.98±1.42	1.44±0.07	8.58±0.46	1031.69±63.65	190.90±10.80
AKB-11	20.17±1.15	1.26±0.05	13.00±0.60	1199.43±70.28	119.28±7.36
AKB-12	22.13±1.36	0.82±0.02	16.11±1.12	2005.93±147.23	89.45±5.25
AKB-13	11.76±0.66	1.14±0.06	15.90±0.90	442.12±28.11	88.51±4.39
AKB-14	19.98±1.16	0.63±0.03	1.83±0.11	232.95±18.21	42.55±2.39
AKB-15	5.48±0.28	0.57±0.03	6.86±0.35	760.48±49.27	39.88±2.48
AKB-16	38.48±1.97	1.62±0.08	10.29±.58	2539.88±138.16	93.01±5.67
AKB-22	35.61±2.31	0.75±0.04	6.99±0.48	295.31±23.03	33.60±1.93
AKB-23	10.40±0.62	0.56±0.03	1.12±0.06	173.80±10.70	28.96±2.22
AKB-24	59.20±3.50	1.06±0.05	2.40±0.13	1648.80±104.86	72.80±3.87
AKB-25	24.00±1.05	1.10±0.05	27.80±1.37	1214.20±69.69	152.10±8.53
AKB-26	9.43±0.48	0.87±0.04	24.80±1.51	355.63±20.19	54.48±3.35

Note: gds= Gram dry substrate

Table 3. Use of crude enzymes from different fungal enzymes for bio-deinking of MOW and hydrolysis of pearl millet stover

After pulping (blank)				Reducing sugars after hydrolysis of pearl millet stover (mg/g of substrate)
Brightness	59.7			
After flotation				
Fungal isolates	Brightness (%)	Deinking efficiency (%)	Reducing sugars in filtrate	
AKB-10	70.7±0.3	59.48±1.7	0.30±0.02	205.15±9.23
AKB-11	67.5±0.3	42.18±1.6	0.23±0.03	230.24±9.67
AKB-12	71.2±0.1	62.18±0.8	0.28±0.01	224.83±8.86
AKB-13	68.4±0.2	47.04±1.1	0.33±0.04	237.66±5.70
AKB-14	70.7±0.1	59.48±0.9	0.29±0.03	90.71±3.45
AKB-15	69.3±0.2	51.91±1.2	0.22±0.03	110.38±5.08
AKB-16	67.2±0.3	40.56±1.6	0.39±0.02	268.45±14.23
AKB-22	69.8±0.3	54.61±1.7	0.21±0.02	254.74±13.76
AKB-23	71.0±0.2	61.10±1.2	0.26±0.03	97.16±4.37
AKB-24	71.2±0.2	62.18±1.4	0.25±0.01	114.35±5.37
AKB-25	71.3±0.1	62.73±0.8	0.34±0.01	280.27±9.67
AKB-26	70.4±0.2	57.86±1.1	0.29±0.04	210.25±8.83

3.2.1 Pulping conditions

Pulping time 20 min, Surfactant (w/w) 0.10%, pH 9.5±0.2, Consistency 10%, Temperature 40ºC

3.2.2 Enzymatic treatment conditions

Reaction time 60 min, Enzyme dose 2 IU/g, pH 5.0±0.2, Consistency 10%, Temperature 50±2ºC

3.2.3 Flotation conditions

Flotation time 10 min, Surfactant (w/w) 0.10%, pH 7.5±0.2, Consistency 1%, Temperature 35±2ºC

3.3 Enzyme Production

Enzyme production was carried out by the above selected two fungal strains, *Aspergillus nidulans* AKB-25 and *Penicillium* sp. AKB-24. *Aspergillus nidulans* AKB-25 produced maximum endoglucanase (152.14±8.29 IU/gds), FPase (3.42±0.18 FPU/gds), xylanase (2441.03±144.99 IU/gds) and amylase (37.41±1.95 IU/gds) under optimum conditions using black gram (*Vigna mungo*) residue as substrate. *Penicillium* sp. AKB-24 produced maximum endoglucanase (133.94±7.19 IU/gds), FPase (2.96±0.14 FPU/gds), xylanase (3592.26±170.99 IU/gds) and amylase (194.90±11.05 IU/gds) under optimum conditions using wheat bran as the substrate [28].

3.4 Characterization of Cellulases from *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24

3.4.1 pH characterization of cellulases

Optimum pH for FPase, endoglucanase, and glucosidase activities was 5.0 for both the fungal strains i.e. *A. nidulans* AKB-25 and *Penicillium sp.* AKB-24. The fungal isolates i.e. *A. nidulans* AKB-25 and *Penicillium sp.* AKB-24 exhibited broad range of pH (3.0-7.0) for FPase, endoglucanase, and β-glucosidase activities. Fig. 2 reveals that 65.83, 63.81 and 51.34% of FPase, endoglucanase, and β-glucosidase respectively were retained at pH 3.0 and 43.15, 58.54 and 66.47% of FPase, endoglucanase, and β-glucosidase were retained at pH 7.0 for *A. nidulans* AKB-25. For fungal strain *Penicillium* sp. AKB-24 FPase, endoglucanase, and β-glucosidase retained 77.53, 88.06 and 89.19% of activities respectively at pH 3.0 and 47.19, 71.64, and 64.43% of FPase, endoglucanase, and β-glucosidase activities respectively were retained at pH 7.0 (Fig. 3). The optimum pH 5.0 for both the fungal strains may also be suitable for deinking of rosin-alum sized waste paper which have the pH range 4.3-4.8 during paper making. Tavares et al. [29] found an optimal pH of 4.0 for endoglucanase from *Aspergillus nidulans*. An optimum pH of 5.0 have been reported for endoglucanases from *Aspergillus fumigatus* Z5 [30] and *Penicillium pinophilum* MS 20 [31].

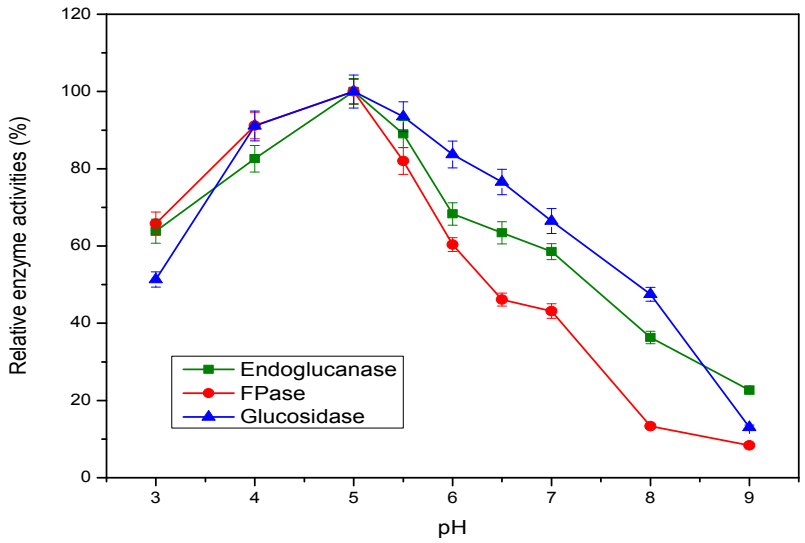

Fig. 2. Effect of pH on cellulase activity by *Aspergillus nidulans* AKB-25 at 50°C

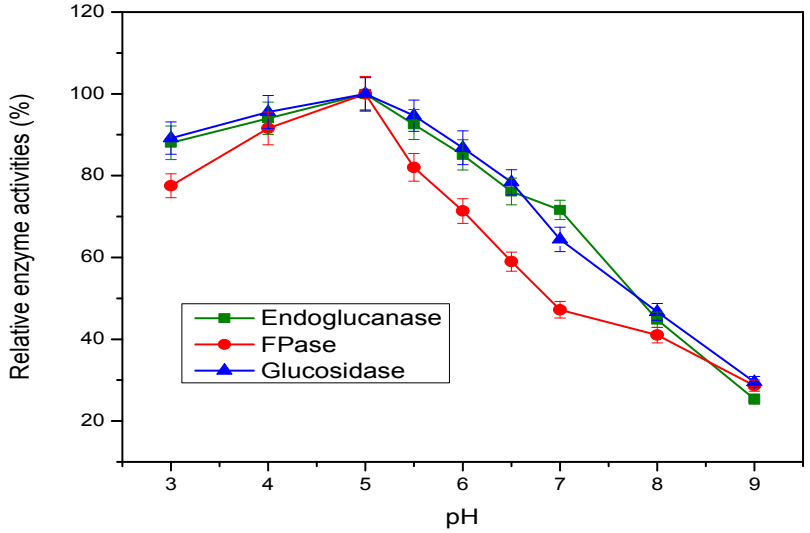

Fig. 3. Effect of pH on cellulase activity by *Penicillium* sp. AKB-24 at 50°C

3.4.2 Temperature characterization of cellulases

Cellulases from *A. nidulans* AKB-25 and *Penicillium sp.* AKB-24 exhibited quite good FPase, endoglucanase, and glucosidase activities over a broad temperature range of 40-75ºC. Endoglucanase from *A. nidulans* AKB-25 showed good activity at temperature ranging from 40-75ºC, with maximal activity at 65ºC and retained 68.87% endoglucanase activity at 75ºC while maximum FPase activity was observed at 60ºC and 30.41% FPase activity was retained at 75ºC. From the same fungal strain, optimum β-glucosidase activity was found at 60°C and 56.02% β-glucosidase activity was retained at the 75ºC. Cellulases from *A. nidulans* AKB-25 were found moderately thermo-stable with optimum endoglucanase activity at 65ºC and optimal FPase and β-glucosidase activities at 60ºC (Fig. 4). Endoglucanase activity from *Penicillium* sp. AKB-24 increased with increasing temperature up to 55ºC, but beyond that declined progressively and retained 60.27% of activity at 70ºC. The maximal FPase and β-glucosidase activities were observed at 55ºC and retained 38.18 and 36.37 % of FPase and β-glucosidase activities at temperature 70ºC for fungal strain *Penicillium sp.* AKB-24 (Fig. 5).

Tavares et al. [29] reported optimum endoglucanase activity at 40ºC from *Aspergillus nidulans*. Liu et al. [30] reported the maximum endoglucanase activity at 50ºC from *Aspergillus fumigatus* Z5. Saini et al. [16] found optimum cellulase and β-glucosidase from *Penicillium oxalicum* at temperature 50 and 60ºC respectively and 53 and 65% of cellulase activities remained at 40 and 60ºC respectively.

3.4.3 Thermostability and pH stability of cellulases

Thermostability and pH stability of enzymes from *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24 were studied at different temperatures, holding times and optimum pH (Table 4). Endoglucanase and FPase activities at temperature 25°C and 50°C decreased slightly with increasing holding time on the other hand, at 60°C, endoglucanase activity reduced drastically after 24 h of holding time and FPase activity after 3 h for fungal strain *A. nidulans* AKB-25. Endoglucanase and FPase produced by *A. nidulans* AKB-25 had half life of more than 22 and 3 h respectively at 60°C. When the holding time increased, the decline in glucosidase activity increased with increasing temperature (Table 4).

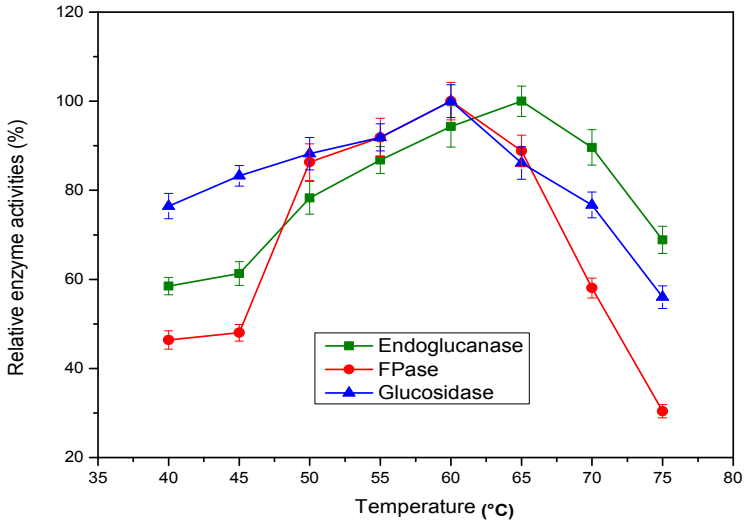

Fig. 4. Effect of temperature on cellulase activity by *Aspergillus nidulans* AKB-25 at pH 5.0

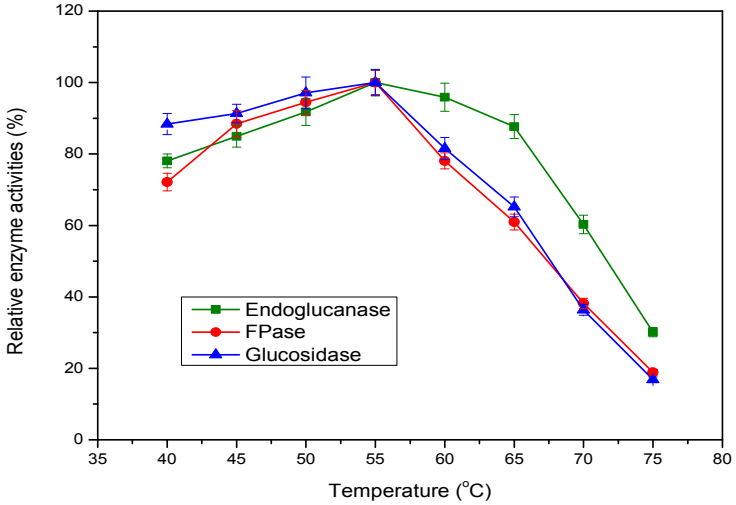

Fig. 5. Effect of temperature on cellulase activity by *Penicillium* sp. AKB-24 at pH 5.0

The glucosidase activity reduced rapidly after 3 h at 60ºC for fungal strain *A. nidulans* AKB-25. β-glucosidase from *A. nidulans* AKB-25 showed approximately 2 h of half life at 60ºC. Likewise, endoglucanase, FPase and β-glucosidase activities decreased with increasing temperature at different holding time for *Penicillium* sp. AKB-24. The endoglucanase, FPase and β-glucosidase activities at 55ºC were only 44.61, 24.13 and 22.81% respectively after 24 h of holding time. Endoglucanase and FPase produced by *Penicillium* sp. AKB-24 showed half life of approximately 21 and 6 h respectively at 55ºC. β-glucosidase from *Penicillium* sp. AKB-24 was retained 90.49% of relative activity after 48 h of holding time at 50ºC while 54.36% of β-glucosidase was retained after 48 h of holding time for *A. nidulans* AKB-25. β-glucosidase from *Penicillium* sp. AKB-24 showed approximately 12 h of half life at 55ºC. Tavares et al. [29] also found that endoglucanase activities from *Aspergillus nidulans* at 45 and 55ºC up to 72 h were not affected significantly. Castro et al. [32] showed that cellulases from *Penicillium funiculosum* were highly stable at 37ºC up to 23 h. β-glucosidase and endoglucanase retained 56 and 53% of their activities at 50ºC after 23 h. However, cellulases from *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24 were stable at 50°C up to a holding time of 48 h. The prolonged stability of cellulases makes them suitable for saccharification of cellulosic biomass for a long time, without any extra requirement of enzymes. The stability of enzyme for a longer time at higher temperature is desirable features for industrial applications [29].

3.4.4 Partial purification of endoglucanases

Enzymes produced at optimum culture conditions by both the fungal strains *A. nidulans* AKB-25 and *Penicillium sp.* AKB-24 were subjected to ammonium sulphate precipitation (Table 5). Maximum endoglucanase activities were recorded at 40-70% and 30-70% ammonium sulphate for *A. nidulans* AKB-25 and *Penicillium sp.* AKB-24 respectively. The fraction of 40-70% ammonium sulphate precipitation gave yield of 68.10% with 3.63 fold purification for *A. nidulans* AKB-25. The fraction of 30-70% ammonium sulphate precipitation resulted yield of 60.33% with 2.88 fold purification for *Penicillium sp.* AKB-24 (Table 5). Ammonium sulphate precipitation has been used widely for partial purification of enzymes and at saturation level of 30-70% has been used for the purification of cellulases and xylanases from various microbial sources [33-36].

Table 4. Thermo and pH stability of cellulases from *Aspergillus nidulans* AKB-25 and *Penicillium* sp. AKB-24 at different temperatures and pH 5

		***Aspergillus nidulans* AKB-25 (Relative activity, %)**								
		Endoglucanase			**FPase**			**β-glucosidase**		
Temperature		**60°C**	**50°C**	**25°C**	**60°C**	**50°C**	**25°C**	**60°C**	**50°C**	**25°C**
Time(h)	Control	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1	98.43	98.33	100.0	83.26	95.88	99.86	61.20	96.62	95.11
	3	96.87	98.33	98.46	49.45	93.21	99.56	37.35	70.08	90.26
	6	93.75	96.66	96.92	21.34	91.57	96.52	13.81	63.69	82.74
	24	46.87	96.66	95.38	16.83	91.16	93.65	3.65	58.22	72.25
	48	-	95	95.38	-	88.82	92.50	-	54.36	64.34
		***Penicillium* sp. AKB-24 (Relative activity, %)**								
Temperature		**55°C**	**50°C**	**25°C**	**55°C**	**50°C**	**25°C**	**55°C**	**50°C**	**25°C**
Time(h)	Control	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1	93.84	96.96	100.0	83.99	98.15	100.0	89.92	97.16	100.0
	3	87.69	95.42	98.25	65.94	95.53	99.20	77.14	95.44	98.61
	6	80.00	95.42	96.72	50.53	95.05	95.68	65.19	94.54	95.94
	24	44.61	92.34	95.18	24.13	93.71	93.03	22.81	91.77	94.74
	48		90.80	92.11	-	93.65	88.94	-	90.49	91.14

Table 5. Partial purification of cellulases from *Penicillium* sp. AKB-24 *and Aspergillus nidulans* AKB-25

Fungal strain	Step	Volume (mL)	Activity (IU/mL)	Total activity (IU)	Protein (mg/mL)	Total protein (mg)	Specific activity (IU/mg)	Yield (%)	Fold purification
Penicillium sp.	Crude enzyme	200.00	11.47	2294.00	3.59	718.00	3.19	100.0	1
	$(NH_4)_2SO_4$ (70%)	14.25	97.12	1384.03	10.53	150.10	9.22	60.33	2.88
Aspergillus nidulans	Crude enzyme	200.00	13.69	2738.00	2.78	556.00	4.92	100.0	1
	$(NH_4)_2SO_4$ (70%)	10.50	177.60	1864.80	9.93	104.26	17.88	68.10	3.63

Table 6. Effect of metal ions on endoglucanase activity of *Aspergillus nidulans* AKB-25 and *Penicillium* sp. AKB-24

Metal ions	Relative endoglucanase activity (%)			
	Penicillium sp. AKB-24		*Aspergillus nidulans* AKB-25	
	1 mM	10 mM	1 mM	10 mM
Na^{+}	100.54±2.21	98.63±3.36	99.73±2.45	99.99±3.45
K^{+}	104.65±2.66	102.73±2.84	98.42±3.32	97.90±2.84
Al^{+++}	102.19±3.17	99.45±2.61	84.29±4.06	79.31±3.25
Ca^{++}	107.94±3.37	105.20±4.36	99.73±2.10	100.26±3.31
Cd^{++}	90.95±2.78	86.84±3.67	93.45±3.60	92.14±2.87
Co^{++}	84.10±2.72	79.72±3.40	80.10±3.76	67.53±3.19
Cu^{++}	68.21±3.12	13.67±0.66	98.16±3.12	66.49±2.95
Fe^{++}	98.35±2.42	77.26±3.18	96.59±3.16	76.17±3.71
Hg^{++}	0	0	0	0
Mn^{++}	64.93±3.19	61.91±2.92	95.02±3.04	90.05±2.47
Mg^{++}	100.82±3.18	99.99±2.34	95.81±3.54	94.24±3.15
Ni^{++}	109.58±3.56	106.02±3.35	97.38±2.14	94.76±3.26
Pb^{++}	96.71±2.20	78.08±3.09	91.36±4.39	79.31±3.78
Zn^{++}	118.95±4.08	124.79±5.92	111.78±2.24	122.54±2.72
Control	100.00±3.38	100.00±3.27	100.00±2.84	100.00±3.33

3.4.5 Effect of cations on partially purified cellulases

Cellulose pulp and process water may contain a number of metal ions, which in turn may affect the enzyme activity during bio-deinking of waste paper or saccharification process. With this in mind, the effect of metal ions on endoglucanase activities was examined. Metal ions such as, Zn^{2+} had the stimulatory effect, which enhanced the relative endoglucanase activity by 124.79 and 122.54% respectively for both the fungal strains (Table 6). These ions may bind to the enzyme, causing conformational changes that result in an increased enzyme activity. 1 and 10 mM concentrations of Hg^{++} strongly inhibited the endoglucanase activity for both the fungal strains. In earlier studies, increase in endoglucanase by Zn^{++} has been reported by several researchers [31]. Hg^{++}, Cu^{++}, Co^{++}, and Mn^{++} are commonly cited in literature as inhibitors for several microbial cellulases [37-39]. Enzyme activity is probably inhibited through attack on certain groups at the active sites of enzymes. Inactivation of endoglucanase by Cu^{++} and Hg^{++} probably indicate presence of thiol and histidine as active site residues [31].

4. CONCLUSIONS

Fungal isolate AKB-24 and AKB-25 were selected based on their effectiveness in deinking of MOW and saccharification of pearl millet stover. The optimum pH for FPase, endoglucanase, and glucosidase activities was 5.0 for both the fungal strains i.e. *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24. The optimum temperature for endoglucanase activity was 65ºC while optimum temperature for FPase and β-glucosidase activities was found 60ºC for

fungal strain *A. nidulans* AKB-25. The maximal endoglucanase, FPase and β-glucosidase activities were observed at 55ºC for fungal strain *Penicillium* sp. AKB-24.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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