ENVIRONMENTALLY FRIENDLY TOTALLY CHLORINE FREE BLEACHING OF WHEAT STRAW PULP USING NOVEL CELLULASE-POOR XYLANASES OF WILD STRAINS OF *COPRINELLUS DISSEMINATUS*

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Cellulase-poor crude xylanases of *Coprinellus disseminatus*, strains SH-1 NTCC 1163 (enzyme-A) and SH-2 NTCC 1164 (enzyme-B) produced under optimum conditions of solid-state fermentation (SSF), were used in bio-bleaching of wheat straw soda-AQ pulp in totally chlorine free (TCF) bleaching sequences. Kappa number reductions of 56% and 58% with respect to oxygen-delignified pulps were obtained after the sequences OX^AE and OX^BE, respectively. Significant increases in pulp brightness of 6.07% (enzyme-A) and 3.34% (enzyme-B) with slight decreases in some strength properties (due to the removal of hemicelluloses) were observed. Removal of hemicelluloses was further validated by an increase in pulp viscosity (6.07%, and 4.58%), COD (40%, and 38%), and facilitation of lignin removal, as indicated by colour values (48% and 45%) for OX^AEQPP and OX^BEQPP bleached pulps, respectively, over the control. Crude xylanases from *C. disseminatus* SH-1 NTCC 1163 and SH-2 NTCC 1164 can successfully be used for TCF bleaching of pulps owing to their high temperature and pH toleranance, and cellulase-poor nature, thus adding to the search for environment-friendly bleaching solutions for the pulp and paper industry.

Keywords: Coprinellus disseminatus; Wheat straw; Pulp and paper; TCF bleaching

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INTRODUCTION

Fast dwindling of forests has prompted interest in renewable resources for obtaining industrial products, such as chemicals, pulp, and fuels. Agricultural residues are emerging as a significant alternative raw material resource for the pulp and paper industry, where wheat straw constitutes the major part of cereals' residues, and a sizeable portion is available for the pulp and paper industry. The scarcity of paper raw materials has grown in parallel with environmental pollution problems, where the pulping and bleaching processes can cause serious contamination problems. Pulp bleaching is an issue of great concern, primarily because of environmental hazards associated with the use of chlorine-based bleaching processes. This drew the focus on total chlorine-free bleaching processes, which significantly reduce the effluent loadings and allow total system closure. At the same time, the study and optimization of new bleaching sequences is required so that the chemical consumption as a whole can be decreased. This has shifted the focus of research towards the use of biological means for environment-friendly

bleaching processes. Bio-bleaching with highly specific hemicellulolytic enzymes (particularly, endo-1,4-β-xylanases) before chemical pulp bleaching is now practiced at a commercial scale of application. The use of xylanases with a totally chlorine free (TCF) bleaching sequence can substantially improve the bleachability of conventional pulps, thereby increasing the final brightness ceiling (Allison et al. 1994). Only a few studies have been made on the hemicellulases of white-rot fungi, and relatively little is known about them. This notwithstanding, it is clear from their ability to decay wood, and from studies demonstrating growth on hemicelluloses substrates, that white-rot fungi have effective hemicellulase systems (Tenkanen et al. 1996).

The present investigation assesses the potential of cellulase-poor crude xylanases of novel strains of *C. disseminatus*, SH-1 NTCC 1163 and SH-2 NTCC 1164 (Singh et al. 2009) for bio-bleaching of soda-AQ pulp of wheat straw in TCF bleaching. The efficacy of the same in conventional bleaching has been proved (Singh et al. 2010).

EXPERIMENTAL

Organism and Enzyme Production

Novel strains SH-1 NTCC 1163 and SH-1 NTCC 1164 of *Coprinellus disseminatus* were purified on wheat bran agar medium (2% wheat bran and 2% agar-agar) and incubated at 37ºC. Purified cultures were routinely cultured on potato-dextrose-agar (PDA) slants at 37ºC for 72 h and subsequent storage at 4ºC. The cultures were preserved as a suspension of spores and hyphal fragments in 15% (*v*/*v*) sterile glycerol stored at -20ºC. The test strains were subjected to optimized conditions of solid state fermentation (SSF) (Singh et al 2009) to obtain crude xylanases for bio-bleaching.

Enzyme Assays

Xylanase activity was determined by taking 1.6 mL of appropriately diluted enzyme preparation (the filtrate after removing pellets). This was added in a sterile tube, which contained 0.4 mL of substrate suspension (10 mg/mL of birchwood xylan in 0.1M potassium phosphate buffer to get a pH level of 6.0). The assay mixtures were incubated for 15 min at 55 ºC with a constant shaking at 100 rpm. The reducing sugars liberated were measured by the DNS method at 540 nm (Miller 1959) and expressed as xylose equivalent. One unit of activity was defined as the amount of enzyme needed to release 1 µmol of xylose equivalents released per min at 55 ºC. Carboxymethylcellulase (CMCase) activity was determined by incubating 2 mL of enzyme preparation with 2 mL of 2% (*w*/*v*) carboxymethylcellulose (CMC) as the substrate, procured from Qualigens Fine Chemicals, Mumbai (Mandels 1975), prepared in 0.05 M citrate buffer (pH 4.8) at 50 ºC for 30 min. The reducing sugars were measured by the DNS method (Miller 1959) at 575 nm and expressed as glucose equivalent. The enzyme activity was expressed as µmoles of D-glucose equivalents released per min at 50 ºC and pH 4.8 (IU).

Laccase activity was determined by the continuous spectrophotometric rate determination method (Ride 1980) with syringaldazine as the substrate. Sets of test samples containing 2.2 mL of reagent ‘A’ (100 mM potassium phosphate buffer, pH 6.5 at 30 ºC) and 0.5 mL reagent ‘C’ (enzyme solution prepared at suitable concentration in

cold deionized water), and blank solutions containing 0.5 mL deionized water and 2.2 mL reagent 'A' were pipetted into suitable cuvettes. These cuvettes were equilibrated to 30 ºC. Absorbance at a wavelength at 530 nm (A_{530} nm) was monitored until constant, using a thermostatted spectrophotometer. After equilibration, 0.3 mL of reagent 'B' (0.216 mM syringaldazine solution) was added to each test sample and the blank solution. The contents in each cuvette were immediately mixed by inversion, and the increase in absorbance at 530 nm ($A_{530\ nm}$) was recorded for about 10 min. The change in $A_{530\ nm}$ per min was obtained by using the maximum linear rate for both the test and blank. One unit is defined as unit that will produce $A_{530\ nm}$ of 0.001 per min at pH 6.5 at 30 ºC in a 3 mL reaction volume using syringaldazine as the substrate and the enzyme activity was expressed as unit per mL of the sample.

The maximum xylanase activity was at 55^{o}C for both the strains. Enzyme-A retained 43 and 12% of its activity for SH-1 and enzyme-B retained 25 and 20.22% of its activity for SH-2 at 65 and 75^{0}C, respectively. Xylanase activity was the highest at pH 6.4 for both the strains. For SH-1, enzyme-A retained 49.03, 32.64 and 5.70% of its activity and for SH-2, enzyme-B retained 95.30, 35.03 and 5.60% of its activity at pH 7.0, 8.0 and 9.0, respectively. At pH 6.0, the enzyme retained 97.47 and 89.48% of its activity for strains SH-1 and SH-2, respectively (Singh et al. 2009). It showed the thermo-tolerant and alkalo-philic nature of both the strains and could successfully be applied for industrial applications as the pulp produced after brown stock washing has high temperature (about 68^{0}C) and alkaline in nature (pH about 8.1) (Agnihotri et al. 2010).

Pulp Sample

Wheat straw soda-AQ pulp of screened pulp yield 45.05% and kappa number 18.25 obtained under optimum pulping conditions of soda-AQ pulping was used for bio-bleaching (Singh et al. 2011).

Pulp Bleaching

The wheat straw pulp was first O_2 delignified at a pressure of 5 kg/cm^2, NaOH 2% (as Na_2O), $MgSO_4$ (a carbohydrate stabilizer) 0.1%, temperature 110ºC, time 90 min, consistency 15%, and pH 11.0 in a CCL digester (Feronics, Roorkee, India), and evaluated for kappa number (T236 cm-85) (Anonymous 2007) after washing. Enzyme treatment was applied after oxygen delignification (Blanco et al. 1995) under optimized conditions of enzyme dose (10 IU/g), reaction time (3 h), and pulp consistency (10% for enzyme-A and 5% for enzyme-B) at a temperature of 55^{o}C and a pH of 6.4 (Singh et al. 2010). Pulps were washed with tap water after enzyme treatment for proper impregnation of subsequent bleaching chemicals (Zhao et al. 2002). The enzyme-treated pulps were extracted with 2% NaOH (as Na_2O) at 70±2 ^{o}C for 90 min and pH 11.0, followed by chelation (Q stage) at 1% EDTA, pulp consistency 3%, time 30 min, pH 5.5 and temperature 50±2 ºC. The pulps were subjected to two consecutive peroxide stages (P_1 and P_2) using 1.5% peroxide charge for each 'P' stage, at pulp consistency 10%, temperature 90±2 ºC, time 2 h, and pH 11.8. Similarly, control pulp was bleached with an OQPP sequence. The pulps obtained after different bleaching stages of respective bleaching sequences were filtered through cheese cloth and filtrates were collected.

Residual peroxide of respective stages of concerned bleaching sequence was determined immediately. The pulps were washed with 2 L of tap water. The remaining filtrates and the washed pulp samples were stored in the dark at 4 ºC for further analysis.

Characterization of Pulps and Pulp Bleaching Effluents

Thick pulp pads were prepared (T 218 sp-02) and evaluated for kappa number (T 236 cm-85), brightness (ISO) (T 452 om-02), and CED viscosity (T 230 om-04) (Anonymous 2007). Pulps were beaten (T 248 sp-00) at a beating level of 40±1 ^{o}SR in a PFI mill at a consistency of 30±1%. Laboratory hand sheets (60 g/m^2) were prepared on a British sheet former (T 205 sp-02), conditioned at a relative humidity of 65%±2 and temperature 27±1 ºC, and evaluated for burst index (T 403 om-02), tensile index (T 494 om-01), double fold (T 423 cm-98), and tear index (T 414 om-04) (Anonymous 2007). The filtrates from different stages of the bleaching sequence were separately mixed in equal amounts, and the combined effluents were analyzed for COD and color. COD was determined by the closed reflux titrimetric method using a Thermoreactor CR2010 (Training manual, E. Merck (I) Ltd and WTW instructional manual, Weilheim, Gmbh), and colour by the cobaltiplatinate method at 465 nm using UV-VIS spectrophotometer (Systronics UV Visible 118). One unit equals to the absorbance produced by 1 mg/mL of platinum present in the form of cobaltiplatinate ion at 465 nm.

Statistical Analysis

For brightness, six experimental values, and for kappa number and viscosity three experimental values, in each case, were taken. The results are mean ± standard deviation (SD) of the values.

RESULTS AND DISCUSSION

Organism and Enzyme Production

Crude xylanases obtained from *Coprinellus disseminatus* SH-1 NTCC 1163 (enzyme-A) and *Coprinellus disseminatus* SH-2 NTCC 1164 (enzyme B) under optimum SSF conditions (Singh et al, 2009) were used for bio-bleaching. Under optimum conditions, the enzyme-A showed 727.78 IU/mL xylanase activity, 0.925 IU/mL cellulase activity, and 0.640 U/mL laccase activity, while enzyme-B showed 227.99 IU/mL xylanase activity, 0.660 IU/mL cellulose activity, and 0.742 U/mL laccase activity.

Pulp Bleaching

The results for bio-bleaching are reported in Table 1. Oxygen treatment reduced pulp kappa number by 45.2%. The enzyme treatment mitigated the kappa number of O^2-delignified pulp by 20.2% and 24.1%, respectively, indicating that enzyme-B was better than enzyme-A in reducing the pulp kappa number. The decrease in kappa number occurred due to enzyme treatment, which is expected if xylanase attacked LCC or xylan associated with lignin, thus, liberating lignin (Ragauskas et al. 1994). The brightness of OX^AEQPP and OX^BEQPP bleached pulps, respectively, improved by 6.0 and 3.3%, respectively, as compared to the control (OQPP).

Table 1. Effect of Enzyme Treatment on OQPP Bleaching of Soda-AQ Pulp of Wheat Straw

Particulars	Bleaching sequences		
	OQPP	**OX^{A}EQPP**	**OX^{B}EQPP**
Oxygen (O) stage§			
Pressure, kg/cm^2	5	5	5
NaOH, %	2	2	2
MgSO4, %	0.1	0.1	0.1
Kappa number after oxygen delignification	10.0 ± 0.14	10.0 ± 0.14	10. ± 0.14
Enzyme dose, X*, IU/g§	—	10	—
Enzyme dose, X**, IU/g§	—	—	10
Extraction (E), NaOH applied§**, %**	—	2	2
Kappa number after alkali extraction	—	8.0 ± 0.11	7.6± 0.10
Chelation (Q) stage, EDTA applied§**, %**	1	1	1
Peroxide (P) stage			
Total peroxide charge§**, %**	3	3	3
P_1 stage			
H_2O_2 applied, %	1.50	1.50	1.50
H_2O_2 consumed, %	1.49	1.47	1.46
P_2 stage			
H_2O_2 applied, %	1.50	1.50	1.50
H_2O_2 consumed, %	1.49	1.46	1.46
NaOH, %	2	2	2
Sodium silicate, %	5	5	5
$MgSO_4$, %	0.05	0.05	0.05
Final brightness, % (ISO)	65.80 ±0.3	69.80 ±0.24 (+6.07)	68.00 ±0.22 (+3.34)
Final viscosity, cps	10.04 ± 0.04	10.65± 0.07 (+6.07)	10.50± 0.03 (+4.58)
Pulp beating level, ^{0}SR	40±1	40±1	40±1
Mechanical strength properties			
Tensile index, Nm/g	50.60	42.28 (—16.44)	42.51 (—15.98)
Burst index, kPa m^2/g	3.42	3.01 (—11.98)	3.04 (—11.11)
Double fold, no.	67	60 (—10.44)	61(—8.95)
Tear index, mNm^2/g	5.36	5.53 (+3.17)	5.57 (+3.91)
Combined bleach effluent properties			
COD, mg/L	1200	1680.00 (+40.00)	1656 (+38.00)
Colour, PCU	100.10	148.14 (+48.00)	145.14 (+45.00)

Bleaching conditions	**X^A**	**X^B**	**E**	**O**	**Q**	**P_1**	**P_2**
pH	6.4	6.4	11.0	10	5.5	11.8	11.8
Consistency, %	10	5	10	15	3	10	10
Retention time, min	180	180	90	90	30	120	120
Temperature, ^{0}C	55±2	55±2	70±2	110±2	50±2	90±2	90±2

X^A = Enzyme-A, X^B = Enzyme B, Chemical charge on o.d. pulp basis, (+/—) = % difference compared to control pulp, ± = Standard deviation from the mean, Pulp beating level = 40±1 ^{0}SR, Unbleached pulp kappa number = 18.25, Unbleached pulp brightness, % (ISO) = 27.41, Unbleached pulp viscosity, cps = 26.04.

The viscosity of OX^AEQPP and OX^BEQPP pulps, respectively, was found to improve by 6.07 and 4.58%, compared to OQPP bleached pulps. The increase in viscosity might be due to the selective removal of lower DP (degree of polymerization) xylan and consequent enrichment of high molecular weight polysaccharides (Kantelinen 1993). The tensile indexes for OX^AEQPP and OX^BEQPP pulps decreased by 16.44% and 15.98%, respectively, while the burst indexes for the same were lowered by 11.98% and 11.11%, respectively, as compared to the control. Xylanase-treated wheat straw pulps have, on average, longer fibers and lower fines content than the control, which are disadvantageous for fiber bonding (Zhao et al. 2002). The removal of hemicelluloses and the concomitant reduction in DP of residual hemicelluloses might reduce tensile and burst indexes. The double fold for OX^AEQPP and OX^BEQPP pulps was 10.44% and 8.95%, respectively, while tear index for the same pulps improved by 3.17% and 3.91%, respectively, as compared to control. The increase in tear index indicates that though excess xylan removal reduced burst and tensile strength by reducing inter-fiber bonding, the fibers themselves were not weakened (Roberts et al. 1990).

The combined bleach effluent of OX^AEQPP and OX^BEQPP pulps showed an increase in COD (40% and 38%, respectively) and colour (48% and 45%, respectively) compared to OQPP pulp. Enzyme action weakens pulp carbohydrate bonds and causes its dissolution. This increases the concentration of lignin and hydrolyzed xylan in the effluent, leading to increased COD and colour (Roncero et al. 1996; Vidal et al. 1997).

CONCLUSIONS

The crude cellulase poor xylanases obtained from novel strains SH-1 NTCC1163 and SH-2 NTCC1164 of *Coprinellus disseminatus* can be successfully applied in total chlorine free pulp bleaching, thereby adding to the search of environmentally friendly solutions for pulp and paper industry.

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