BIO-CONVENTIONAL BLEACHING OF KRAFT-AQ PULP OF *A. CADAMBA* BY CRUDE XYLANASES FROM *COPRINELLUS DISSEMINATUS* MLK-03 AND EFFECT OF RESIDUAL ENZYME ON EFFLUENT LOAD

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A new thermo-alkali-tolerant crude xylanase from *Coprinellus disseminatus* decreased kappa number by 34.38% and improved brightness and viscosity by 1.6 and 6.47% respectively after XE_1-stage during prebleaching of *Anthocephalus cadamba* kraft-AQ pulp. At 2.4% chlorine demand, crude xylanase in a XECEHH (X= enzymatic prebleaching stage, E= extraction stage, C= chlorination stage, H= hypochlorite stage) bleaching sequence improved pulp brightness, tensile index, burst index, and double fold numbers by 3.66%, 4.78%, 6.38%, and 11.11%, respectively with a reduction in viscosity (10.59%) and tear index (10.77%) compared to the control. Combined bleach effluent of the XECEHH sequence mitigated adsorable organic halides (AOX) by 21% and increased chemical oxygen demand (COD), bio-chemical oxygen demand (BOD), and colour by 67.18%, 84.78%, and 97.53%, respectively, compared to the control. Residual enzymes that entered during enzymatic prebleaching stage decreased AOX, COD, BOD, and colour of combined effluent of the XECEHH bleaching sequence progressively and on 6th day, and these were reduced by 23.78%, 0.04%, 15.00%, and 0.61%, respectively, compared to the control.

Keywords: Anthocephalus Cadamba; Coprinellus disseminates; CEHH bleaching; Pulp and paper; Residual enzymes; Effluent characteristics

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INTRODUCTION

Bleaching of pulp uses large amounts of chlorine-based and other chemicals, which cause several effluent-related problems in the pulp and paper industries (Beg et al. 2001). The chlorination stage is the first stage in which the congeners 2,3,7,8-TCDD (tetrachlorodibenzo-p-dioxin), 2,3,7,8-TCDF (tetrachlorodibenzofuran), and 1,2,7,8-TCDF are always present. The filtrate of the alkali extraction stage has the highest concentrations of dioxins and furans, which can change blood chemistry, and cause liver damage, skin disorders, lung lesions, and tumours (Norseth 1997). Use of such type of a paper product for direct body contact consumables (baby diapers), food packaging (tea bag paper), bread and biscuits wrappers, and crimped and curd cups used in railway catering services and confectioners to hold the better class sweetmeats, and crystallized fruits and agro-farming research (seed germination paper) is of major concern, since it is associated with chlorinated compounds that are carcinogenus in nature (Dutt et al. 2004,

2005a, 2005b, 2007). In response to governmental directives and environmental protection groups, paper industries are presently changing practices to minimize the use of chlorine-based chemicals. The available options for attaining the above objectives are oxygen delignification, bio-pulping, substitution of ClO_2 for Cl_2, and use of H_2O_2 and O_3 (Beg et al. 2001; Nasman et al. 2007). Most of these methods involve high capital investment. Thus, an alternative and cost-effective method is the use of xylanases, which has provided a very simple and economic way to reduce the use of chlorine and other bleaching chemicals compared to oxygen delignification or substitution of ClO_2 for Cl_2 or use of H_2O_2. This makes it possible to reduce the amount of toxic compounds (chlorophenols and other forms of organically bound chlorine) in the spent bleach liquor (Jain et al. 2006). The absence of cellulase, as well as high stability under the high temperature and various levels of pH used for treatment are the prerequisite characteristics of xylanases, enabling them to be used in the pulp and paper industry. Treatment with xylanases boosts the overall bleaching process by improving subsequent stages (Valls and Roncero 2009; Shatalov and Pereira 2007; Roncero et al. 2003) and has even been incorporated into bleaching sequences in some pulp mills. The enzymatic treatment with oxidoreductases such as laccases is a promising alternative under intensive research. These enzymes are capable of oxidizing phenolic units and amine compounds in lignin (Higuchi 2004). In combination with redox mediators, laccases can expand their action to non-phenolic substrates (Freudenreich et al. 1998). Several studies have confirmed the potential of the so-called laccase-mediator system (LMS) for the bleaching of different types of pulp (Ibarra et al. 2006; Sigoillot et al. 2005). Singh et al. (2009) observed that under optimum SSF conditions of incubation (period 7 days, temperature 37^{o}C, pH 6.4, carbon source wheat bran, and organic nitrogen source yeast extract), the strain SH-1NTCC 1163 of *Coprinellus disseminatus* yielded xylanase, CMCase, and laccase activities of 727.78 IU/mL, 0.925 IU/mL, and 0.640 U/mL, and the same for strain SH-2 NTCC 1164 yielded 227.99 IU/mL, 0.660 IU/mL and 0.742 IU/mL, respectively (Singh et al. 2009). The xylanase and laccase activities of *C. disseminatus* SW-1 NTCC 1165 under optimum solid-state fermentation conditions using wheat bran and soya bean meal as carbon nitrogen sources respectively at incubation period (7 days), temperature (37^{o}C), and pH (6.4) were 499.60 IU/mL and 25.5 IU/mL, respectively along with negligible cellulase contamination (0.86 IU/mL) (Agnihotri et al. 2010). Two novel cellulase-poor xylanases from *Coprinellus disseminatus* SH-1 NTCC-1163 (enzyme-A) and SH-2 NTCC-1164 (enzyme-B) produced under solid-state fermentation mitigated kappa number of wheat straw soda–AQ pulps by 24.38% and 27.94%, respectively after XE-stages. ^{A}XECEHH and ^{B}XECEHH sequences improved brightness by 5.17 and 2.58% respectively at 4.5% chlorine charge. AOX in ^{A}XECEHH and ^{B}XECEHH sequences was reduced by 56.11% and 55.75% respectively at 4.5% chlorine charge and 68.34% and 67.98% respectively at 2.25% chlorine charge respectively compared to control (Singh et al. 2011).

The present communication aims at investigating pre-bleaching of kraft-AQ pulp of *A. cadamba* with thermo-alkali-tolerant xylanase from *Coprinellus disseminatus* MLK-03 produced under solid-state fermentation (SSF) conditions in CEHH bleaching sequence, and its effect on pulp brightness, pulp viscosity, mechanical strength properties, and effluent characteristics including colour, COD, BOD, and AOX were

evaluated. The combined effluent of XECEHH bleaching was analyzed to observe the effect of residual crude xylanase on AOX, COD, BOD, and colour after the 1st, 2nd, 4th, and 6th day of incubation.

EXPERIMENTAL

Microorganism and Enzyme Production

An alkali-tolerant strain of *Coprinellus disseminatus* was isolated from a decaying wood sample by enrichment culture technique in which the decaying wood sample was kept in glass petri plate containing moist wheat bran. This was incubated at 40 ^{o}C, and growth was observed every day. After the appearance of white-rote basidiomycetes, this was purified on wheat bran agar medium, containing 2% wheat bran, and agar powder each without any nutrients by frequently subculturing. The purified culture was stored in 20% glycerol at -20 ^{o}C, and subcultured after an interval of 3 to 4 months. Xylanase was produced under SSF conditions. 15 g of wheat bran powder was taken in a 500 mL Erlenmeyer flask in which 45 mL of nutrient salt solution (NSS) containing 1.5 g/L KH_2PO_4, 4.0 g/L NH_4Cl, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, and 1.0 g/L yeast extract with 0.4 mL/L trace element solution (FeSO4.7H2O 200 µg/L, MnSO4 180 µg/L and ZnSO4.7H2O 20 µg/L) was added. The pH of NSS was adjusted at pH 8.0 before autoclaving with 1.0N NaOH/HCl. These were autoclaved at 121^{0}C for 15 min. Two disks of 8 mm diameter from 4-5 day old culture of white-rote basidiomycete were inoculated with help of sterile cork borer in each flask, and these were incubated at 40^{0}C in a BOD incubator for 7 days. After 7 days of incubation, the flasks were harvested in 45 mL distilled water, and contents were filtered through 4 layered cheese cloth followed by centrifugation at 5000 rpm for 10 min in Remi Centrifuge. This crude enzyme was used in biobleaching experiments without further purification.

The xylanase activity was determined by measuring the release of reducing sugars using birch wood xylan (Sigma Chemicals Co.) as substrate by (3, 5-dinitrosalicylic acid) (DNS) method (Miller 1959) at 55^{0}C for 15 min with constant shaking at 100 rpm. Optical density was measured at 540 nm in a double beam UV-visible spectrophotometer. The enzyme activity is expressed as µmoles of D-xylose equivalents released per min at 55 ^{o}C (IU). The cellulase activity in terms of CMCase was determined as described above except using 2% CMC (carboxylmethylcellulose) as a substrate in place of xylan. Lignin peroxidase activity was measured as described by Mercer et al., using 2,4-di-chlorophenol as a substrate at 510 nm (Mercer et al. 1996). The enzyme activity was expressed as the amount of enzyme produced with an increase of 1.0 absorbance unit per 30s. Laccase activity was determined by continuous spectrophotometric rate determination (Ride 1980). One unit is defined as unit producing a 530 nm of 0.001 per min at pH 6.5 at 30 ^{o}C in a 3 mL reaction volume using syringaldazine as substrate. Enzyme activity was expressed as IU/mL of the sample. *C. disseminatus* MLK-03 has xylanase activity 38.90 IU/mL, cellulase activity 0.28 IU/mL, Lignin peroxidase activity, 0.25 IU/mL and laccase activity 0.75 IU/mL at pH 8.0 and temperature 75^{0}C.

Pulp Characteristics

The screened chips of *A. cadamba* (age 12 years) were digested in a WEVERK electrically heated rotary digester of capacity 0.02 m^3 by kraft pulping process at an active alkali dose of 16% (as Na_2O), sulphidity 20%, maximum cooking temperature 165^0C, time (at temperature) 90 min and liquor to wood ratio of 3.5:1. The screened pulp yield of *A. cadamba* is found to be 48.74% at kappa number of 16±0.40, pulp brightness 35±0.32% (ISO) and viscosity 27.8±0.26 cps (Lal et al. 2010).

Conventional (CEHH) Bleaching Sequence

The *A. cadamba* kraft-AQ pulp was pre-bleached pulp with crude xylanase from *C. disseminatus* MLK-03 under optimum prebleaching conditions, i.e. an enzyme dose of 5 IU/g (oven dry pulp basis), consistency 10%, reaction time 120 min, and temperature 75 ^{o}C. Subsequently, the prebleached pulp was extracted with 2% NaOH at 70 ^{o}C and 10% consistency for 90 min. The pulp was filtered through cheese cloth, and the filtrates were collected for further analysis. Then, the pulp was washed with tap water. The enzyme treated pulp after XE-stage was evaluated for kappa number (TAPPI T236 cm-85 "Kappa number of pulp"), brightness (TAPPI T452 om-02 "Brightness of pulp, paper and paperboard [Directional reflectance at 457 nm]"), and viscosity (TAPPI T206 os-63 "Cupprammonium disperse viscosity of pulp") (Anonymous, 2007). Now the pulp was bleached by CEHH bleaching sequence at 4.0% total chlorine demand. 2.4% molecular chlorine was applied in chlorination stage and 0.80% calcium hypochlorite as available chlorine in H_1 and H_2 stages respectively. The process conditions for each stage are reported in Table 1. The results of XECEHH bleaching sequence was compared with the results of CEHH bleaching sequence. Pulps obtained after each bleaching stage was filtered through cheesecloth, and filtrate was analyzed for residual chlorine, except for the alkali extraction stage. The rest of the filtrates were preserved at 4 ^{o}C for further analysis, and the pulps were washed with 2 L of tap water, squeezed, and crumbled.

Beating, Sheet making and Evaluation of Physical Strength Properties

Pulps were beaten (TAPPI T 248 sp-00 "Laboratory beating of pulp [PFI mill method]") at fixed beating level (35 ^{0}SR) in PFI mill. Laboratory handsheets (60 g/m^2) were prepared on British sheet former (TAPPI T205 sp-02 "Forming handsheets for physical tests of pulp"), conditioned at relative humidity 65%±2 and temperature 27±1ºC and evaluated for burst index (TAPPI T 403 om-97 "Bursting strength of paper"), tensile index (TAPPI T494 om-01 "Tensile properties of paper and paperboard [using constant rate of elongation apparatus]"), double fold (TAPPI T423 cm-98 "Folding endurance of paper [Schopper type tester]") and tear index (TAPPI T414 om-98 "Internal tearing resistance of paper [Elmendorf-type method]") (Anonymous 2007). Thick pulp pads were prepared (TAPPI T218 sp-02 "Forming handsheets for reflectance testing of pulp [Büchner funnel procedure]") and evaluated for brightness (TAPPI T452 om-02 "Brightness of pulp, paper and paperboard [Directional reflectance at 457 nm]"), and viscosity (TAPPI T206 os-63 "Cupprammonium disperse viscosity of pulp") (Anonymous 2007).

Characterization of Bleach Effluent

The bleach effluent generated from each stage of bleaching sequence was mixed in equal amounts and were analyzed for COD ((IS3025: Part 58, 2006: COD- Closed reflux titrimetric method using Thermo reactor CR2010)), BOD (IS3025: Part 44: 2006- and colour), as per Bureau of Indian Standards for methods of sampling and test (Physical and Chemical) for water and wastewater and AOX by column method (User manual ECS 1200 Rev. 3.1.0, 2006) with AOX Analyzer Dextar ECS 1200. The effect of residual enzymes present in combined effluent of XECEHH bleaching of *A. cadamba* kraft-AQ pulp on COD, BOD, colour, and AOX was studied. The combined effluent was kept during study period as such in laboratory at room temperature varying from 30 to 35^0C in 24 h.

RESULTS AND DISCUSSION

Prebleaching of kraft-AQ pulp of *A. cadamba* with xylanase from *C. disseminatus* MLK-03 followed by alkali extraction mitigated kappa number by 25% after XE stage (Table 1). The brightness and viscosity after XE-stage were improved by 1.5 and 10.7%, respectively. The pulp brightness was improved because crude xylanase improved the accessibility of bleaching chemicals by disrupting the xylan chain and thus, facilitated the easier removal of lignin during bleaching (Senior and Hamilton 1992). Viscosity increase may be due to selective removal of lower degree of polymerization (DP) of xylan, and enrichment of high molecular weight polysaccharides, as observed by Kantelinen et al. (1993) and Paice et al. (1992). The brightness of XECEHH bleached pulp at 2.4% chlorine charge increased by 3.00%, and pulp viscosity dropped by 10.59%. There was no adverse effect of CMCases present in the crude enzymes, as viscosity drops when cellulases cleave cellulose chains, lowering the DP of cellulose, and destroying fiber integrity (Kirk and Jeffries 1996). A mild cellulase activity might improve pulp fibrillation and induce better fibre bonding (Maiti and Whitmore 1997). It can be seen by the observation that XECEHH bleached pulp required 45.20% less PFI revolutions to achieve a 35 ^{o}SR compared to CEHH bleached pulp. This indicates that cellulase contamination present in enzyme preparation swells the fibres and subsequently reduces the refining energy.

Table1 reveals that tensile index, burst index and double folds number of XECEHH bleached pulp increased by 4.78%, 9.61%, and 11.11% respectively compared to CEHH bleaching. On the other hand, the tear index was reduced by 10.77% for XECEHH bleached pulps compared to the control. The tear index is influenced by fiber length, while tensile index, burst index and double folds numbers are influenced both by fiber length and extent of hydrogen bonding. The cellulase present in the enzyme preparation plays an important role not only in reducing of refining energy but improving in mechanical strength properties of enzymatically treated pulps also. Clark *et al.* (1991) suggested that xylanase prebleaching increases the fiber swelling, which facilitates refining and in turn results in better physical properties. The results indicate that xylanase prebleaching can have facilitated an increase in pulp fibrillation, water retention, restoration of bonding, and freeness in fibers (Battan et al. 2007). For tear energy, the

necessary work that has to be done to pull the fibers loose depends on the length of the fibers as well as the bond strength. As a result of improved fibrillation due to enzyme action, the inter-fiber bond strength will be higher and fibers start to break instead of being pulled out intact. It generally takes less work to break a fiber than to pull it out.

Table1. Effect of *C. disseminates* MLK-03 Treatment on Kraft-AQ Pulp of *A. cadamba* during CEHH Bleaching

Parameters	Bleaching sequences	
	CEHH	XECEHH
Unbleached pulp kappa number	16±0.20	
Unbleached pulp brightness, % (ISO)	35±0.32	
Unbleached pulp viscosity, cps	27.8±0.26	
Enzyme treatment followed by alkali extraction with 2% NaOH (XE_1)		
Pulp kappa number	–	12±0.12 (–25)
Pulp brightness, % (ISO)	–	36.5±0.23(+1.5)
Pulp viscosity, cps	–	30.6±0.25 (+10.7)
Chlorination (C) stage		
% Cl_2 applied as avail Cl_2 (on od pulp basis)	2.40	2.40
% Cl_2 consumed as avail Cl_2 (on od pulp basis)	2.38	2.12
Alkali extraction (E_2)		
%, NaOH applied on oven dry pulp basis	1.20	1.20
Brightness, % (ISO)	46.4±0.32	50.6±0.33
Hypochlorite (H_1) stage		
% $Ca(OCl)_2$ applied as avail Cl_2 (on od pulp basis)	0.80	0.80
% $Ca(OCl)_2$ consumed as avail Cl_2 (on od pulp basis)	0.79	0.78
Hypochlorite (H_2) stage		
% $Ca(OCl)_2$ applied as avail Cl_2 (on od pulp basis)	0.80	0.80
% $Ca(OCl)_2$ consumed as avail Cl_2 (on od pulp basis)	0.78	0.75
Final brightness, % (ISO)	81.9±0.28	84.9±0.26 (+3.00)
Final viscosity, cps	8.5±0.12	7.6±0.10 (–10.59)
Pulp beating		
Number of revolutions to achieve 35 ^{0}SR	950	520 (–45.20)
Mechanical strength properties		
Tensile index, Nm/g	56.50	59.20 (+4.78)
Burst index, kPa m^2/g	5.81	6.38 (+9.61)
Tear index, mNm^2/g	6.50	5.80 (–10.77)
Double folds number	36	40 (+11.11)

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Dose
C-stage	Ambient	45	2.5	3	2.4%
E_1 ' E_2 stages	70±2	90	11.4	10	1.2%
H_1, H_2 stages	70±2	90	11.3	10	0.8% + 0.8%
Enzyme	65±2	120	7.5	10	5 IU/g

(+/-) = % difference compared to control pulp, ± = standard deviation

Table 2 shows that AOX in the combined effluent of XECEHH bleached pulp was mitigated by 21% compared to the control. This AOX reduction is remarkable, as AOX reduction of about 20-45% for xylanase treated pulps was reported by Senior and

Hamilton (1952). The combined effluent of XECEHH bleaching was kept at 30 to 35 ^{o}C for six days, and effect of residual crude xylanase on AOX, COD, BOD, and colour was investigated. After 2nd, 4th, and 6th day of incubation AOX in combined bleached effluent of XECEHH sequence was reduced by 1.36%, 2.74%, and 5%, respectively, compared to combined bleach effluent of XECEHH collected on the 1st day, while AOX on the 6th day of incubation was reduced by 23.78% compared to the control. In the same way COD was reduced by 20.95%, 28.14%, and 42.51%, BOD by 27.06%, 41.18%, and 52.94%, and colour by 10.31%, 20.00%, and 49.67% respectively on the 2nd, 4th, and 6th day of incubation compared to values of combined bleach effluent of XECEHH sequence collected on the 1st day of incubation. Likewise, COD, BOD, and colour were mitigated by 0.04%, 15%, and 0.61% on the 6th day of incubation compared to the control. The residual enzyme contains laccase, and lignin peroxidase is capable of oxidizing phenolic units, and amine compounds in lignin. The chromophore release at 237, 280, and 465 nm confirms that crude xylanase containing xylanase alongwith lignin peroxidase and laccase acts on LCC, releasing degraded chromophores into the effluent. The kappa number decrease after XE-stages (enzyme treatment followed by alkali extraction) can be attributed to facilitation of dissolution of lignin-carbohydrate fragments that were previously modified by these enzymes but still remained in pulp because of their large molecular weight (Singh et al. 2011).

In combination with redox mediators, laccases and lignin peroxidase can expand their action to non-phenolic substrates (Ibarra et al. 2006; Sigoillot et al. 2005). In black liquor treatment with *T. versicolor*, Font et al. (2003) detected laccase activity but no LiP or MnP activities, suggesting that laccase may be largely responsible for the removal of phenols in mill effluents treated with *T. versicolor*. *Phanerochaete chrysosporium* reduces COD by degrading the chlorolignin to CO_2 and chloride, decolorizes the bleaching effluent by destroying both the colour bodies and chromophoric structures and removes total organic chlorides (TOCl) by converting them to inorganic chloride (Chang et al. 1987; Eaton 1985).

Table 2. Effect of Residual Enzyme on Combined Effluent Generated during XECEHH Bleaching Sequence on COD, BOD, AOX, and color.

Parameters	CEHH	XECEHH	Incubation periods, days		
			2	4	6
AOX, kg/T	1.85	146 (–21%)	1.44 (–1.36%)*	1.42 (–2.74%)*	1.41 (–23.78%) (–5.0%)*
COD, mg/L	999	1670 (+67.18)	1320 (–20.95%)*	1200 (–28.14)*	960 (–0.04%) (–42.51%)*
BOD, mg/L	230	425 (+84.78)	310 (–27.06%)*	250 (–41.18%)*	200 (–15%) (–52.94%)*
Colour, PCU	810	1600 (+97.53)	1435 (–10.31%)*	1280 (–20.0)*	805 (–0.61%) (–49.67%)*

*Values compared to XECEHH, rest values compared to control

CONCLUSIONS

Crude xylanases obtained from *C. disseminates* MLK-03 were found to be thermo-stable and alkalophilic, along with other accessory enzymes like laccase, lignin peroxidase, and with minimum contamination of cellulase. This enzyme may successfully be applied to pulp produced after brown stock washing, which has high temperature (about 70^0C) and alkaline in nature (pH about 8.5). *C. disseminates* MLK-03 is able to improve the brightness of pulp (84.9%), and reduce the refining energy (45.20%) compared to a control. All the mechanical strength properties are improved except tear index. AOX in combined bleached effluent (XECEHH) is reduced by 21% compared to CEHH bleaching sequence. However, COD, BOD, and colour are increased due to dissolution of lignin carbohydrate complex. The accessory enzymes present in crude xylanase are also able to reduce AOX by 23.78%, COD 0.04%, BOD 15%, and colour 0.61% on the 6^{th} day compared to the control. Application of enzyme in prebleaching stage may be helpful to small scale paper industry, which does not have chemical recovery plant. The combined effluent was collected in lagoon for precipitation of lignin during lignin recovery process (LRP). Residual enzymes left after bleaching may get sufficient time for incubation. After LRP, effluent is subjected to be treated by activated sludge basis treatment for final discharge.

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