Bacterial cellulase treatment for enhancing reactivity of pre-hydrolysed kraft dissolving pulp for viscose

Chakarvati Sango¹ · Prabhjot Kaur² · Nishi K. Bhardwaj² · Jitender Sharma¹

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Abstract

To improve the process economy of reactivity improvement, crude cellulase from *Bacillus subtilis* was employed for the treatment and significant dissolving pulp properties were analyzed. With increase in enzyme dose from 0.25 to 2 U/g o.d. pulp, improvement in Fock reactivity and alkali solubilities (S10 and S18) were observed with simultaneous reduction in viscosity and yield. Fourier transform infrared spectroscopy and scanning electron microscopy were used to observe the molecular level effects on dissolving grade pulp. The most suitable cellulase dose for reactivity improvement with lowering of viscosity was 0.25 U/g o.d. pulp. With increases in enzyme dose, alkali solubilities (S10 and S18) of dissolving pulp showed continuous increment, while alpha-cellulose of pulp showed reduction due to chain scission of long cellulose fiber fraction.

Keywords Dissolving pulp · Crude cellulase · Reactivity · Viscose · Alkali solubility · *Bacillus subtilis*

Introduction

Dissolving pulp is chemically synthesized pulp which consists of 90–98% cellulose with 2–10% of hemicelluloses, residual lignin, minerals, and extractives. Dissolving pulp is a key material for the manufacture and production of rayon, cellophane, cellulose acetate, cellulose ethers, etc. (Bajpai 2012; Kaur et al. 2016a, b). Approximately 70% of dissolving pulp is used in the viscose process for cellulosic fiber (Bajpai 2012; Sixta et al. 2013).

Production of pre-hydrolysis kraft pulp has been carried out through alkaline cooking which includes extraction of hemicellulose from wood chips in a pre-extraction step. However, the main drawback of this process is the formation of sticky precipitates due to the synthesis of highly reactive intermediates during condensation reactions (Annergren et al. 1965; Sixta et al. 2006; Leschinsky et al. 2008a). To overcome this problem, a process named steam reactivation is carried out during the neutralization process prior to alkaline cooking (Wizani et al. 1994).

The critical parameter for production of viscose rayon is the reactivity of dissolving pulp, also termed as Fock reactivity (Roffael 1988; Sixta et al. 2004; Filpponen and Argyropoulos 2008; Köpcke 2010). Fock reactivity is the capacity of the pulp to react with carbon disulphide under defined conditions (Christofferson et al. 2002; Strunk 2012). It indicates the processability of dissolving pulp for viscose and measures the percentage of reacted cellulose during the xanthation reaction. To improve the reactivity of pulp, cellulases and their monocomponents have been widely used for the treatment of different hardwood and softwood pulps (Rahkamo et al. 1998). Also, the upgradation of paper pulp, dissolving pulp using enzymes for improvement in pulp properties, has been studied extensively (Jackson et al. 1998). Cellulases are hydrolytic enzymes that cleave 1,4- β -D-glucosidic linkages of the cellulose chain (Zhang and Lynd 2004). Cellulase consists of three enzymes, i.e., endoglucanase, β -glucosidases, and cellobiohydrolases in which endoglucanases have been used specifically for cellulase reactivity enhancement of dissolving pulp (Henriksson et al. 2005; Engström et al. 2006; Kvarnlöf 2007; Köpcke 2010). Cellulase treatment causes the formation of additional openings in fiber structure, resulting in increased pore volume of pulp fiber and enhancement of its reliability to the xanthation process (Miao et al. 2015). The hydroxyl groups attached to C(2), C(3), and C(6) are the main reactive groups susceptible

✉ Jitender Sharma
jksharmakuk@rediffmail.com

¹ Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana 136 119, India

² Avantha Centre for Industrial Research and Development, Paper Mill Campus, Yamuna Nagar, Haryana 135 001, India

to chemical modification such as acetylation, xanthation, and nitration (Kaur et al. 2016a, b). Improvement in the reliability of the Fock method has been obtained by modification in its procedure (Tian et al. 2013).

Higher cellulose reactivity in the viscose process causes homogeneous substitution in the xanthation process leading to better quality viscose product. Also, higher cellulose reactivity causes more cellulose dissolution in a lesser amount of the hazardous carbon disulphide (CS_2) solution which leads to less environment pollution (Kvarnlöf et al. 2007). Viscosity of pulp is an important parameter for characterization of pulp which refers to the length of the cellulose chain. It gives analysis of degree of polymerisation of cellulose or relative indication in degradation of cellulose fiber. For improvement in cellulose reactivity, many studies have been organized on the usage of cellulase enzymes (Wang et al. 2014; Miao et al. 2014; Duan et al. 2016). In this present study, the crude cellulase enzyme from *Bacillus subtilis* was investigated at different enzyme doses for improvement in cellulose reactivity of pre-hydrolysed kraft pulp. The advantage of using crude enzyme lies in the fact that the purification cost is not involved which improves the process economy.

Experimental

Materials

The hardwood pre-hydrolysed kraft dissolving pulp [*Eucalyptus globulus* (55–60%), *Leucaena leucocephala* (30–25%), *Casuarina equisetifolia* (20–10%)] was a kind gift from a mill in Hyderabad, India. Prior to cellulase treatment, the air-dried sheets of dissolving pulp were soaked overnight and disintegrated.

Crude cellulase production

The strain was isolated from soil and identified by 16S rRNA from Bhat Biotech, Bangalore. Crude cellulase enzyme was produced from *Bacillus subtilis* in Erlenmeyer flasks (250 ml) containing 50 ml of basal medium having (g/l): yeast extract, 5.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; KH_2PO_4 , 1.0 supplemented with rice bran, 5.0 and peptone, 5.0; and pH 9.0. The flasks were inoculated with 0.5% of the 6-h grown inoculum and incubated at 37 °C under shaking conditions (180 rpm) for a 42-h incubation period. The enzyme was harvested by centrifuging at 10,000×g for 15 min. The enzyme activity of crude cellulase was 12.94 IU/ml as assayed by Bailey's method (Bailey et al. 1992). The crude enzyme extract contains cellulase as well as traces of xylanase enzymes. There

was no significant effect of xylanase on brightness of dissolving pulp.

Determination of Fock reactivity

Dissolving pulp reactivity refers to the dissolution of cellulose fiber in carbon disulfide (CS_2) during xanthation and was measured by the modified Fock method (Fock 1959; Kaur et al. 2016b). For reactivity determination, “0.50 g o.d. (oven dry)” pulp sample was taken with dryness no less than 90%. The handsheets were torn into 1 × 1 cm pieces and put into 250-ml capped Erlenmeyer flasks. The pieces were completely wetted and later flooded with 50 ml of 9% (w/w) NaOH (sodium hydroxide) solution. Then the alkali-immersed pulp was kept for shaking in an incubator shaker at 25 °C for 5 min. After that, 1.3 ml of CS_2 was incorporated and the flasks were tightly capped and closed with paraffin wax. The xanthation was accomplished for 3 h at 25 ± 2 °C in an incubation facility shaker at 250 rpm. The reaction was terminated by the addition of deionized water to get the weight of the reaction mixture up to 100 g and by vivacious shaking to get a homogeneous reaction mixture. For the separation of undissolved cellulose and reacted cellulose, the reaction mixture was centrifuged at 5000 rpm for 10 min. The clear supernatant (10 ml) was then pipetted out to a 100-ml Erlenmeyer flask and neutralized with 3 ml of 20% (w/w) H_2SO_4 (sulfuric acid). For the degassing of separated cellulose, the mixture was kept overnight at room temperature. The regenerated cellulose was then acidified with 20 ml of 68% (w/w) H_2SO_4 and mixed for 1 h on a magnetic stirrer. The mixture was then diluted to make up 50 ml final volume and proceeded for oxidation in a 250-ml flat-bottom round container. Approximately 10 ml of 1 N potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) was then incorporated and the reaction mix was refluxed for 1 h. The reaction mixture was cooled down to room temperature and diluted to 100 ml total volume in a volumetric flask. Then 40 ml of solution was drawn from it and 5 ml of 10% potassium iodide (KI) was incorporated. The iodine released was titrated with 0.1N sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) using 5% starch as an indicator. For the confirmation of cellulose (%) that reacted with carbon disulfide, volume (ml) of $\text{Na}_2\text{S}_2\text{O}_3$ consumed for each sample pulp was recorded. Each experiment was done in triplicate and average value is given.

Evaluation of other pulp properties

Cellulase treatment is a well-known method to bring about modifications in cellulose fiber and its supramolecular structure that impact different dissolving pulp properties. To evaluate the impact of crude cellulase treatment,

significant dissolving pulp properties such as alkali solubility, viscosity, and alpha-cellulose were assessed by utilizing TAPPI standard techniques. Viscosity that indicates the degree of polymerization of the cellulose was determined using TAPPI standard method T 230 om-08 (2013). This method describes a procedure for determining the viscosity of 0.5% cellulose solutions, using 0.5 M cupriethylenediamine as a solvent and a capillary viscometer.

Alkali solubility represents the dissolution of low-DP (degree of polymerization) cellulose content of pulp in different concentrations of sodium hydroxide solution of 10, 18, or 21.5% at 25 °C for 1 h. The dissolved carbohydrates are determined by oxidation with potassium dichromate. S10 and S18 were measured using TAPPI standard method T 235 cm-09 (2009). Alpha-cellulose that presents undamaged long-chain cellulose content was analyzed using TAPPI standard method T 203 cm-99 (2009). This method involves extraction of pulp with 17.5 and 9.45% sodium hydroxide solutions at 25 °C. The soluble fraction, consisting of beta- and gamma-celluloses, is determined volumetrically by oxidation with potassium dichromate, and the alpha-cellulose, as an insoluble fraction, is derived by difference.

Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM)

The control dissolving pulp and crude cellulase-treated pulps were converted into the handsheets using the Büchner funnel procedure, per TAPPI test method T218 sp 11 (2011). All handsheet samples were analyzed by ATR (attenuated total reflectance) using Frontier MIR LiTa/KBr/Al spectrometer (PerkinElmer, UK) at a resolution of 4 cm⁻¹, in the range of 4000–400 cm⁻¹ wavelength with 32 scans. The crystal used in the ATR cell (PIKE Technologies) was a diamond with refractive index of 2.4. Incident radiation at an angle of 45° was applied. The SEM analysis of the handsheet samples of control and cellulase-treated pulps samples was done by coating the handsheet samples with 5-nm-thick gold film and observing under a scanning electron microscope (JEOL JSM-6510 LV).

Results and discussion

Chemical analysis of pre-hydrolyzed kraft dissolving pulp

Cellulase treatment is preferable for increasing cellulase reactivity as it reduces the crystallinity of the structure by chain scission. This involves dissolution of H-bonds and exposes hydroxyl groups in cellulose structure. Exposure of hydroxyl groups increases the reactivity of cellulose due to their participation in derivatization reactions. The

Table 1 Characterization of pre-hydrolyzed kraft dissolving pulp (initial values of various pulp properties)

Chemical properties	Value
Fock reactivity	53.8%
Viscosity	5.18 cP
α-Cellulose	93.5%
S10	10.66%
S18	4.56%
S10–S18	6.10%

pre-hydrolyzed kraft dissolving pulp sample was treated with bacterial crude cellulase enzyme at different enzyme doses such as 0.25, 0.5, 1, and 2 U/g o.d. pulp. Fock reactivity of the pulp was found to be 53.9% as given in Table 1. Alkali solubilities S10 and S18 were also determined which provides information about low molecular weight polysaccharides in the pulp. The solubility in 10% sodium hydroxide solution (S10) indicates dissolution of both cellulose and hemicellulose low-DP fraction whereas S18, or solubility in 18% sodium hydroxide, represents only hemicellulose. Viscosity and alpha-cellulose content of the dissolving pulp were also analyzed and are presented in Table 1. The electron micrographs at various magnifications were taken for each sample to elucidate morphological changes followed by crude cellulase treatment of dissolving pulp.

Fock reactivity and viscosity

Endoglucanases (a cellulase component) are mainly responsible for improving pulp reactivity while the viscosity of the pulp decreases simultaneously due to degradation of the cellulose crystalline structure. In various pulping processes, i.e., sulphite softwood dissolving pulp (Kvarnlöf 2007); kraft *B. pendula* pulp (Köpcke 2010); oxygen delignified kraft *Eucalyptus globulus* pulp (Gehmayer et al. 2011; Gehmayer and Sixta 2012); PHK and kraft eucalyptus pulp (Wollboldt et al. 2010); and pre-hydrolysis kraft (PHK) hardwood pulp (Miao et al. 2015), enzyme treatment with commercial cellulases has been used specifically for the improvement of pulp reactivity. In this study, PHK mixed hardwood pulp after treatment with crude cellulase at the dose of 0.25 U/g o.d. pulp showed increased Fock reactivity from 53.8 to 69.5% which satisfies the range of commercially dissolved pulp reactivity, i.e., 60–66%. The reactivity of hardwood dissolving pulp showed enhancement with an increase in enzyme dose while the maximum percent increase was observed at the initial dose of 0.25 IU/g o.d. pulp. At the dose of 0.5 U/g o.d. pulp, reactivity of pulp was 74.5% which increased up to 76.3–77.4% with increases in the enzyme dose from 1 to 2 U/g o.d. pulp, respectively. This study showed similarity with the study on eucalyptus sulphite-based dissolving pulp in which Fock reactivity increased from 65 to 82%

Fig. 1 Effect of different doses of crude cellulase on Fock reactivity of dissolving pulp

Fig. 2 Effect of different doses of crude cellulase on viscosity of dissolving pulp

after treatment of endoglucanase on 30 ECU/g (endocellulase unit per grams) dry pulps for 1 h (Ibarra et al. 2010). In another study, Fock reactivity increased from 47.5 to 87.8% with cellulase treatment in the range of 0–5 U/g o.d. pulp (Miao et al. 2014). Engström et al. (2006) found the 100% Fock reactivity of sulphite-based dissolving pulp with enzyme treatment on 27 ECU/g pulp for 30 min. Tian et al. (2013) also showed improvement in Fock reactivity from 49.3 to 71.8% with 6 min under grinding treatment.

Fock reactivity increased from 48.9 to 70.36% by bacterial crude cellulase enzyme treatment (Fig. 1).

Viscosity of pre-hydrolyzed kraft dissolving pulp showed a decreasing trend with increases in dose of crude cellulase enzyme as shown in Fig. 2. This was due to the dissolution of the cellulose chain by enzymatic actions of endoglucanases. Reduction in viscosity is necessary for maintaining product strength and processing of dissolving pulp (Henriksson et al. 2005; Miao et al. 2015). A decreasing trend of viscosity was observed with increase in enzyme dose with the lowest value observed at the dose of 2 IU/g o.d. pulp. Decrease in viscosity of pulp occurred due to excessive chemical attack on cellulose crystal during prolonged cooking of pulp. Due to shortening of cellulose chain length, increment in accessibility for cellulose reactions occurs which might be the reason for increasing fock reactivity and decreasing viscosity of the dissolving pulp.

Effect on alkali solubility and alpha-cellulose

Other properties of the dissolving pulp include alkali solubility (S10 and S18) and alpha-cellulose of pulp after enzymatic treatment. There were increases in alkali solubility with increases in enzyme dose of crude cellulase as shown in Table 2. S10 values indicate dissolution of low-DP polysaccharide content including both degraded cellulose and hemicellulose while S18 shows dissolution of mainly degraded hemicellulose. Differences between S10 and S18 also showed enhancement with increases in enzyme dose. Increases in alkali solubility were accompanied by reduction in alpha-cellulose content that indicated the degradation of long-chain fraction of cellulose due to cellulase action (Table 2). Increase in S10 value of pulp after cellulase treatment may be attributed to hydrolysis of cellulose that resulted in low-DP cellulose (< 150 DP). Increased S18 value indicated increase in degraded polysaccharides (< 50 DP). Similar results were obtained in the study by Kaur et al. (2017) after cellulase treatment of kraft pulp with commercial cellulases.

FTIR analysis

Crude cellulase treatment of dissolving pulp at different doses has resulted in significant differences in the FTIR

Table 2 Effect of different doses of bacterial crude cellulase treatment on various pulp properties

Crude cellulase dose (U/g o.d. pulp)	S10 (%)	S18 (%)	S10–S18 (%)	α -Cellulose (%)
0.25	10.77 $\pm$ 0.2	4.75 $\pm$ 0.3	6.02	93.4
0.50	10.96 $\pm$ 0.5	5.02 $\pm$ 0.3	5.94	93.3
1.0	11.51 $\pm$ 0.4	5.29 $\pm$ 0.4	6.22	92.9
2.0	12.23 $\pm$ 0.7	5.57 $\pm$ 0.3	6.66	92.7

Fig. 3 FTIR analysis of dissolving pulp (C) treated with different doses of crude cellulase

spectrum of each treated pulp (Fig. 3). The intensity of peak at 3335 cm^{-1} and broad absorption band at 3290 cm^{-1} both increased significantly at the increased enzyme dose which is attributed to different $-\text{OH}$ stretching modes and

associated with H-bonding patterns in the polymer. A new peak at 2855 cm^{-1} was observed in 1 and 2 U/g o.d. pulp-treated pulps along with a marked decrease in relative intensity of band at 2901 cm^{-1} , corresponding to the $\text{C}-\text{H}$ stretching vibration. Also, two new peaks at 1540 and 1745 cm^{-1} were observed in crude cellulase-treated pulps representing $\text{C}=\text{C}$ stretching and carbonyl or carboxyl groups, respectively. Pulps with crude cellulase treatment at higher doses showed increased intensity of absorption peak at 1430 cm^{-1} that represents the degree of crystallinity of the cellulose since this band is associated with symmetric CH_2 bending vibration for which it is also known as a crystallinity band (Bhardwaj et al. 2006; Ciolacu et al. 2011). The cellulases preferably attack at amorphous region of cellulose which could be the reason for slight increase in crystallinity. However, crystallinity may decrease also depending upon dose and composition of cellulases. The cellulase treatment of pulp may cause increased exposure of hydroxyl groups due to its etching effect. This also causes the improvement in pulp reactivity. Moreover, the dissolving pulp in the present study contained less than 5% pentosans with negligible lignin. The $\text{C}=\text{C}$ stretching in the pulp may be attributed to residual xylan exposed after cellulase treatment at high dose.

Fig. 4 SEM images at $\times 2500$ of crude cellulase-treated dissolving pulp at the dose **a** 0.25 U/g, **b** 0.50 U/g, **c** 1.00 U/g and **d** 2.00 U/g o.d. pulp

Scanning electron microscopy (SEM)

The scanning electron microscopic images of dissolving pulp after treatment with crude bacterial cellulase are shown in Fig. 4. Cellulases show their hydrolytic potential based on their components. These enzymes cause the hydrolysis of cellulose that enhances its accessibility. Various enzyme doses from 0.25 to 2.0 U/g o.d. pulp were subjected to pre-hydrolyzed dissolving pulp that caused the formation of pores in cellulose fibers as observed under the microscope. This can be attributed to the combined action of endoglucanases and cellobiohydrolases as multiple enzyme systems cause cavity formation in avicel which is predominantly cellulose and comparable to dissolving pulp (Brunecky et al. 2013; Miao et al. 2015).

Conclusions

At the lower dose of 0.25 U/g o.d. pulp, cellulase showed improvement in Fock reactivity of pulp with a simultaneous decrease in viscosity. Even though the high-dose treatment showed maximum value of Fock reactivity, the percent of increase in Fock reactivity was much lower than earlier low doses. This study signifies the potential of bacterial crude cellulase at low doses for increasing the dissolving pulp reactivity and improving pulp properties at the same time.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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