The Effect of Endo-1,4- β -Xylanase From *Thermomyces Dupontii* KKU-CLD-E2-3 on Eucalyptus Pulp Bleaching and Effluent Treatment

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Abstract

Endo-1,4- β -xylanase-chlorine dioxide bleaching of eucalyptus pulp and analysis of effluent was investigated. The eucalyptus pulp bleaching in D0 stage was prepared with enzyme dose 100 Unit of 10% consistency and incubated at 70 °C for 30 min. The brightness level was achieved up to 72.50 (% ISO). The kappa number and viscosity of eucalyptus pulp was found to be 1.70 and 8.90 (cp), respectively. The paper from pulp bleaching with enzyme has shown an increase in brightness, breaking length, bursting strength, and tearing resistance to be 89.60% IOS, 37.27 Nm/g, 138.81 kPa and 218.02 mN, respectively. The SEM and FTIR analysis of pulp fibers revealed a significance of morphological and structural changes. The analysis of the effluent also showed statistically-significant differences in TOC, BOD, TDS and TSS. The FTIR analysis of the effluent showed the organic compounds and chloride dioxin in cases of those treated by chemicals.

Introduction

Eucalyptus pulp bleaching in industry normally uses chemicals in the bleaching process to remove lignin in pulp fiber. Although this process increases the brightness of the pulp, it provides low yield and causes serious pollution. The chlorinated lignin and the polyphenolic intermediates are the major source of environmental pollution which normally found in the effluent from pulp bleaching processes (Haq et al. 2017). The chlorine dioxin is one of the carcinogens causing (indicate the symptoms that dioxin does to human health) in humans if they intake the contaminated water (Germgard and Larsson 1983). This problem can be solved by using xylanase in the bleaching process to reduce the pollution caused by chlorine dioxin with no residues of toxic substances from the manufacturing process (Kumar et al. 2017).

Xylanases (EC 3.2.1.8) are a group of glycoside hydrolase enzymes that can degrade the linear polysaccharide β -1,4-xylan resulting in xylose (Beg et al. 2001). The xylanase cleaves the glycosidic bonds in the xylan backbone, leading to a reduction in the degree of polymerization of the pulp fiber. The mechanism how the enzyme attacks the molecule of xylan depends on the nature of the pulp fiber molecule, such as on the chain length of branching, and the presence of substituents (Walia et al. 2017). Recently, Orozco-Colonia et al. (2019) reported that xylanase could attack the xylan of hemicellulose by inserting between cellulose and lignin resulting in removal of lignin associated hemicelluloses is facilitated with minimal damage of pulp fiber. In addition, pretreatment of pulp with xylanase can decrease the partial disruption of lignin and carbohydrate bonds in pulp fiber, as the result of enhancing the accessibility of the subsequent bleaching chemicals to the pulp (Sridevi et al. 2017). In general, during the process of pulping, bleaching and washing in pulp and paper industry produces effluent that contains with high amount of organic and inorganic compounds including lignin, hemicellulose, cellulose fragments chlorophenols, fatty acids, inorganic salts and the chlorinated dioxins (Sonkar et al. 2019). Thus, the application of xylanase in the pulp and paper industry focuses to reduce the use of chlorine dioxide in pulp bleaching process that decreases the toxicity in effluents. Besides, there was also a report that the effluent produced from paper bleaching by xylanase after pulp washing could reduce the TSS, TDS, TOC, BOD, colour, and pH (Sridevi et al. 2017).

Currently, the pre-treatment of pulp using xylanase has been adopted to be alternate approach that plays an environmental friendly and economically viable method for paper industries. However, in order to use xylanase in the pulp bleaching process, several properties are needed to be considered. The enzyme should be free of cellulase and active at high temperatures. For instance, the enzyme should actually perform at temperature ranging from 50 – 90 °C in alkaline pH (pH 8 – 10) with 3 – 5 h (Techapun et al. 2003). Thermo-alkali-stable xylanase has attracted to use for pulp bleaching which can work well at relatively higher temperatures without additional cooling process requirement. Thus, there is a continuous need to find novel xylanase which is active in alkaline pH and also thermostable during the pulping process. This work presents the application of purified endo-1,4- β -xylanase from *Thermomyces dupontii* KKU – CLD – E2 – 3 in pulp bleaching step under routinely performed by Phoenix Pulp and Paper Public Company Limited in SCG Packaging, Khon Kaen, Thailand, which is tested at alkaline pH and high temperature. Characterization of pulp, paper and effluent after being treated with enzyme and chlorine dioxide bleaching was presented.

Materials And Methods

Pulp and enzyme

Eucalyptus pulp used in the experiment was obtained from press 3 stage of Phoenix Pulp and Paper Public Co., Ltd. in SCG Packaging, Khon Kaen, Thailand. The characteristics of the pulp were as followed: 3.2 kappa number, 53.6% ISO of brightness, 9.4 cp of viscosity and initial pH 9.7. The purified endo-1,4- β -xylanase was produced from *Thermomyces dupontii* KKU-CLD-E2-3 (Accession number LC428093) (Seemakram et al., 2020). The eucalyptus pulp bleaching was carried out by following the method which routinely performed by Phoenix Pulp and Paper Public Company Limited in SCG Packaging.

Effect of endo-1,4- β -xylanase on eucalyptus pulp in Chlorine dioxide first stage (D0)

Eucalyptus pulp was prepared by treating with an enzyme dose 20–100 (U/50 g pulp) in sealed plastic bags with intermittent kneading and 10% (w/v) pulp consistency for a time period of 30 min at 70 °C. Control sample was untreated by enzyme and treated by chlorine dioxide (ClO₂) at a concentration of 15 kg/T pulp which the standard method that factories use to pulp bleaching and the same conditions with an activated enzyme.

Effect of endo-1,4- β -xylanase on eucalyptus pulp in Extraction-Peroxide stage (EOP) and Chlorine dioxide second stage (D1)

After passing the procedure D0, excess enzyme and buffer in eucalyptus pulp were removed by pressing and draining from the pretreated pulp. The eucalyptus pulp samples prepared were treated with 10% (w/v) pulp consistency. The eucalyptus pulp samples were bleached in EOP staged with a NaOH (14 kg/T pulp) and H₂O₂ (5 kg/T pulp) incubated at 65 °C for 120 min. After that, the chemicals in eucalyptus pulp samples were removed by pressing and draining with tap water, and were treated with a ClO₂ (5 kg/T

pulp) of time period for 240 min at 70 °C. Finally, the eucalyptus pulp samples were washed by pressing and draining with tap water again.

Analyses of eucalyptus pulp properties

The eucalyptus pulp bleaching obtained from D0 stage was prepared for hand sheets and analyzed for brightness using a brightness machine (Technidyne Corporation, Color Touch™ 2). The efficiency of the bleaching treatment process was observed for the reduction of kappa number (TAPPI 1990) and viscosity. The eucalyptus pulp samples were dehydrated in acetone, critical point dried and mounted on sample stubs. The feature surfaces of the xylanase-treated and the control samples were observed under scanning electron microscope (SEM) (SEC, SNE-4500M). Fourier-transform infrared spectroscopy (FTIR) (Bruker, TENSOR27) analysis of pulp samples were crushed with potassium bromide (KBr) at room temperature. The peak obtained was compared with standard FTIR spectra.

Characterization of papers

Eucalyptus pulp samples after passing the procedure D1 were used for papermaking at 20 gram to study the properties of the paper. The thickness of the paper was determined according to the method described by Jordan and Popson (1994). The paper was obtained from pulp bleaching with purified xylanase. Thereafter, they were massaged pulp fibers by basket centrifuge at 4500 rpm and then were prepared wet sheets forming by hand sheets with 10% (w/v) pulp consistency. The eucalyptus pulp samples were pressed for consolidation of wet. The paper was baked to remove moisture so that the remaining moisture was about 4–6%, which dried overnight in a temperature-controlled room where it operated at temperature of 25 °C, and the moisture was kept at 55%. The quality of paper was analyzed for their brightness (Technidyne Corporation, Color Touch™ 2), breaking length (REGMED, Horizontal tensile-D-21), bursting tester (ABB AB/Lorentzen & Wettre, RS232C), and tearing tester (Labthink, SLY-S1), respectively.

Effluent characterization of eucalyptus pulp bleaching

The effluent was characterized before disposal by following the standard method described by Sridevi et al. (2017). The enzyme-mediated release of hydrophobic and chromophoric compounds from pulp bleaching process were measured the absorbance at 280 nm and 465 nm, respectively (Manimaran and Vatsala 2007). Total amount of reducing sugars released from pulp bleaching was determined by the DNS method (Miller 1959). The FTIR analysis of effluent after treated eucalyptus pulp by enzyme was carried out. We aim to investigate the functional groups of the contaminant particles obtained during enzymatic treatment into the effluent. The characterization properties of effluent were determined for several parameters including pH, TSS, TDS, BOD (VELP; Scientifica), and TOC (SHIMADZU, SA24).

Statistical analysis

Experimental designs were statistically analyzed by a Completely Randomized Design (CRD) and pair comparisons in the least significant difference (LSD) test to determine variations and the significance of

differences between treatments. All experiments were performed in triplicate, and the results were expressed as the mean. Effects of the variables and the significance of regression coefficients were determined at F-test ($P \leq 0.05$).

Results And Discussions

Effect of endo-1,4- β -xylanase on eucalyptus pulp in Chlorine dioxide stage (D0)

The ability of endo-1,4- β -xylanase from *T. dupontii* KKU – CLD – E2 – 3 in the bleaching of eucalyptus pulp at D0 stage under pulp quality assessment of SCG company was investigated. The results revealed that kappa number significantly decreased up to 46.87% and the brightness increased up to 35.26% after being treated by endo-1,4- β -xylanase dose 100 U, as compared to the untreated control. The chemical pulp bleach can reduce kappa number of 93.75%, resulting in no lignin in the pulp fiber and an increase in brightness of 82.80% ISO (Table 1). The kappa number is an indication of the residual lignin content in pulp fiber which can be used as an indicator of the efficiency of bleaching process (Kumar et al. 2019). A reduction of kappa number and an increase in the percentage of brightness in our experiment was higher than those reported in previous studies. Kumar et al. (2019) reported that the brightness of hardwood pulp pre-treated with xylanase increased 21.56% while the kappa number decreased 20% compared to the untreated samples. According to Wu et al. (2018) who reported that the brightness of eucalyptus kraft was significantly increased about 14.5%, while 24.5% of kappa number was reduced after bleaching by endoxylanase from *Streptomyces griseorubens* LH–3. The viscosity of pulp after bleaching with endo-1,4- β -xylanase dose 100 U was found comparatively higher (9.10 cp) than chlorine dioxide bleached pulp (7.80 cp) (Table 1), which was not significantly different when compared to the untreated case. Higher viscosity means higher cellulose content which is directly proportional to the greater physical strength of pulp (Sharma et al. 2015).

Table 1
Effect of enzyme dose on eucalyptus pulp bleaching in D0 stage.

Conditions	Brightness (%ISO)	Kappa	Viscosity (cp)	Reducing sugar (μ mol/g pulp)	Chromophoric compounds	Hydrophobic compounds
Un	53.60 ^f	3.20 ^a	9.40 ^a	0.00 ^d	1.54 ^d	0.12 ^{cd}
ClO ₂	82.80 ^a	0.20 ^c	7.80 ^b	0.32 ^c	3.56 ^a	0.09 ^d
20 U	55.60 ^e	2.90 ^a	9.10 ^{ab}	1.29 ^{bc}	2.25 ^c	0.20 ^{bc}
40 U	57.70 ^d	2.70 ^a	9.10 ^{ab}	1.29 ^{bc}	3.35 ^b	0.25 ^{ab}
80 U	63.60 ^c	1.90 ^b	9.10 ^{ab}	2.38 ^b	3.43 ^b	0.26 ^{ab}
100 U	72.50 ^b	1.70 ^b	9.10 ^{ab}	13.10 ^a	3.60 ^a	0.33 ^a
CV.	1.30	16.27	9.48	6.41	2.82	2.08
F-Test	**	**	*	**	**	**
Numbers followed by the same letter in the same column are not significantly different according to LSD test ($P \leq 0.05$) (Un = untreated pulp; ClO ₂ = chlorine dioxide-treated pulp similar to the factory condition; 20U-100U = dose of enzyme used to treat pulp, * Significant difference at $P \leq 0.05$, ** Significant difference at $P \leq 0.01$)						

The release of reducing sugar, phenolic compounds and hydrophobic compounds also increased when the enzyme concentration increased (Table 1). The amount of sugar in the effluent of eucalyptus pulp bleaching for xylanase also significantly increased, which shows that the final product from the enzyme treated pulp was released to sugar. The release of reducing sugars, phenolics, and hydrophobic compounds are a result of the reaction of disintegration of lignin and carbohydrate complex from the pulp fibers (Khandeparkar and Bhosle 2007).

Analyses of eucalyptus pulp properties

The surface states of fibers in pulp samples treated with endo-1,4- β -xylanase and chloride were analyzed by SEM. The SEM analysis exhibited noticeable changes on surface fiber in treated enzyme and chloride, compared to the untreated case (Fig. 1). The surface of untreated pulp was smooth and tight (Fig. 1A). Meanwhile the surface of eucalyptus pulp treated by purified xylanase became rough, loose and was in a peeling state and loss in compactness (Fig. 1B). In contrast, the eucalyptus pulp which was attacked by chlorine dioxide became lost in compactness of pulp fibers and more disintegrating (Fig. 1C). This indicated that the enzymatic reaction was more specific to xylan in the pulp fiber more than chemical one. The enzyme treatments may expose cellulose fibers to the surface (Wu et al. 2018) cause surface alteration of the enzyme-treated pulp was due to the activity of xylanase (Raj et al. 2018). Also, the

xylanase activity in the digestion of xylan causes the release of lignin. The pretreatment of eucalyptus pulp using chlorine dioxide caused severe damage to the pulp, in which the strength of fibers was reduced and the cracks and peeling of pulp surface were appeared.

FTIR spectra of untreated, enzyme-treated and chlorine dioxide-treated eucalyptus pulp were compared. The functional group levels were changes in the peaks of modified in pulp samples (Fig. 2). The peaks of FTIR spectra in the pulp fiber were assigned as shown in Table 2. The peak at 1641 cm^{-1} which corresponds to the C = O stretching vibration in conjugated carbonyl of lignin was shown in the untreated and the enzyme-treated pulp samples (Dai et al. 2016), but none in the chemical-treated one. The peaks at 898.19, 665.71 and 617 cm^{-1} corresponds to the C–H stretching of deformation in carbohydrates, and the = C–H and the $\equiv$ C–H bending vibration of alkynes group were only observed in the pulp fiber of untreated and enzyme-treated compared to chlorine dioxide-treated which could not be found.

Table 2
The signals assignment in FTIR spectra of the pulp fiber.

Wave number (cm^{-1})	Assignment
3355–3345	–OH stretching of acid and methanol groups in cellulose
2900–2892	C = H stretching of aliphatic group
1642–1640	C = O stretching vibration in conjugated carbonyl of lignin
1431–1429	O–CH ₃ in-plane vibration in the aromatic ring
1373–1371	C–H stretching of aliphatic in methyl group
1318.01	–CH in-plane bending vibration in cellulose
1164–1161	C–O–C asymmetric stretching vibration
1115–1112	C–O–C stretching of hemicellulose
1061–1057	C–O stretching vibration of cellulose and hemicellulose
1035.11	C–O stretching vibration of lignocellulose
898.19	C–H stretching of deformation in carbohydrates
665.71	=C–H bending vibration of alkenes group
617	$\equiv$ C–H bending vibration of alkynes group

Characterization of papers

Eucalyptus pulp samples after passing through the D1 step were used to prepare papermaking of 20 grams. The quality of paper was analyzed for its brightness, breaking length, bursting strength, and tearing resistance. The examination of the brightness of papers treated by enzyme showed an increase of 29.20% compared to the control, but < 3.45% compared to chlorine dioxide. In addition, the paper samples

from pulp bleached with enzymes treatment showed a maximum value of breaking length, bursting strength, and tearing resistance of 52.25 N m/g, 138.81 kPa and 218.00 mN, respectively, when compared to the untreated control and the chlorine-treated sample (Table 3). The fiber properties of enzyme pre-bleached pulp had a higher value of tear index compared to that of the control, but no significant difference was observed. The tearing index of enzyme-treated pulp increased up to 15.71% which was higher than those from the untreated pulp (Sharma et al. 2014). Obviously, pretreatments by enzyme could improve value tensile, tearing and bursting index better than the use only chemical process.

Table 3
Properties of paper of acquired pulp bleaching with endo-1,4- β -xylanase and chlorine dioxide.

Conditions	Brightness (%ISO)	Breaking length (Nm/g)	Bursting Strength (kPa)	Tearing Resistance (mN)
Un	69.35 ^e	45.79 ^c	119.13 ^b	210.26 ^{ab}
ClO ₂	92.80 ^a	48.99 ^b	112.25 ^c	182.66 ^c
20 U	79.70 ^d	46.97 ^{bc}	119.38 ^b	188.75 ^{bc}
40 U	80.80 ^d	47.01 ^{bc}	123.50 ^b	191.00 ^{bc}
80 U	86.00 ^c	47.74 ^{bc}	124.19 ^b	207.50 ^{ab}
100 U	89.60 ^b	52.25 ^a	138.81 ^a	218.02 ^a
%CV	1.12	5.66	6.40	7.78
F-Test	**	**	**	**
Numbers followed by the same letter in the same column are not significantly different according to LSD test ($P \leq 0.05$) (Un = untreated pulp; ClO ₂ = chlorine dioxide-treated pulp similar to the factory condition; 20U-100U = dose of enzyme used to treat pulp, * Significant difference at $P \leq 0.05$, ** Significant difference at $P \leq 0.01$)				

Effluent characterization of eucalyptus pulp bleaching

The present work revealed that the wastewater obtained after pulp bleaching by endo-1,4- β -xylanase significantly showed lower values of the TSS and TDS of effluents of pulp bleaching for chloride compared to xylanase treatment reduced from 102 to 57 mg/L, and 2183 to 1139 mg/L, respectively. There was also a reduction of TOC and BOD from 60.15 to 6.81 mg/L and 538.00 to 61.33 mg/L, respectively (Table 4). These values are in the range of industrial effluent standards assigned in Thailand (IEST). The TSS, TDS, TOC and BOD in effluent significantly decreased when pulp was bleached by the enzyme compared to using chlorine dioxide. Similarly, Sridevi et al. (2017) reported that the use of xylanase in pulp bleaching process resulted in the reduction of BOD (89.83%), TSS (72.14%) and TDS (53.32%) in the wastewater discharge. The use of our xylanase for pulp bleaching could bring about not only higher quality of effluent which is even better than the IEST, but also reduce the amount of organic/inorganic materials in the effluent. Therefore, the quality of the effluent from xylanase-bleaching

process is ready to be discharged without passing through the company's wastewater treatment processes.

Table 4
Characteristics of the effluent with IEST standards.

Conditions	pH	TOC mg/L	BOD mg/L	TDS mg/L	TSS mg/L
IEST limits	5.5-9.0	-	< 60	2,000	50
Un	9.50 ^c	0.05 ^e	24.00 ^d	410 ^d	35 ^c
ClO ₂	1.72 ^a	60.15 ^a	538.00 ^a	2183 ^a	102 ^a
20	8.50 ^b	2.68 ^b	26.67 ^d	586 ^d	44 ^{bc}
40	8.60 ^b	3.53 ^{cd}	36.67 ^{cd}	729 ^c	47 ^{bc}
80	8.50 ^b	4.84 ^c	53.33 ^{bc}	774 ^c	53 ^b
100	8.40 ^b	6.81 ^b	61.33 ^b	1139 ^b	57 ^b
%CV	1.93	33.21	7.76	52.13	16.73
F-Test	**	**	**	**	**

Numbers followed by the same letter in the same column are not significantly different according to LSD test ($P \leq 0.05$) (Un = untreated pulp; ClO₂ = chlorine dioxide-treated pulp similar to the factory condition; 20U-100U = dose of enzyme used to treat pulp, * Significant difference at $P \leq 0.05$, ** Significant difference at $P \leq 0.01$)

The FTIR spectra of effluents obtained from untreated, enzyme-treated and chlorine-treated samples was compared. The band assignment of the FTIR spectrum of effluent was listed in Table 5. The functional groups remarkable profiles were changed in the wave numbers of 500 to 3900 cm^{-1} as shown in Fig. 3. Interestingly, there were some significant changes at peak around 941.24 cm^{-1} corresponding to C–O stretching of chloride dioxin caused by the combination of Cl and lignin rings which was seen in case of pulp bleaching by chloride only (Gobi et al. 2016), while pulp bleached by endo-1,4- β -xylanase could not be observed. Nevertheless, the chlorine dioxin was shown between 935 and 950 cm^{-1} (Hanley et al. 2015) which caused by holocellulose of pulp from eucalyptus wood with bleached by chlorine dioxide pretreated in pulp bleaching (Bodirlau and Teaca 2007). This provided evidence that endo-1,4- β -xylanase has good exceeding for pulp bleaching compared to commercial chemical treatment. Because in the pulp bleaching process by endo-1,4- β -xylanase can help to improve the quality of pulp and paper. Also, using enzyme has efficiency for economic improvement of effluents and reduces the risk of cancer cause by dioxin for the staffs in working area. Additionally, the odor pollution from the use of chemicals in the factory can be reduced as well.

Table 5
The band assignment of the FTIR spectrum of effluent.

Wavenumber (cm ⁻¹)	Assignment
3800–3557	OH stretching vibration of silanol group
3310–2900	C–H stretching vibration of alkyl group in aliphatic
2358.47	C–O stretching vibration of phosphine
1729.45	C = O stretching of ketone group
1593–1582	C = C stretching, aromatic ring of lignin
1414–1409	OH bending vibration
1356–1396	C–H bending vibration of methyl group
1138.06	C–O stretching of alcohols group
1111.61	C–O–C vibration of ethylene glycol
1045–1039	C–O stretching of ester group
968.74	C = C bending vibration of carboxylic acid group
941.24	C–O stretching of chloride dioxin
849–842	C–H bending of aromatic ring in lignin
877–769	C–H out-of-plane bending of aromatic compounds
699.50	C–Cl stretching
680.21	C–Br stretching of alkyl halides
626–621	C = C bending, out of plane ring

Conclusions

Endo-1,4- β -xylanase bleaching of eucalyptus pulp in D0 stage could increase the paper brightness. The physical properties of the paper including breaking length, bursting strength, and tearing resistance were more improved than those using chemicals bleaching works well in realistic situations. The variety of contaminants present in the effluent from the pulp bleaching process after enzyme bleaching was degraded. Therefore, this revealed environmental benefits than chemical bleaching.

Declarations

Ethics approval and consent to participate

The manuscript represented is not studies involving human participants, human data or human tissue.

Consent for publication

All authors provide consent to publication of the work.

Availability of data and materials

The datasets supporting the conclusions of this article are included in the main manuscript file.

Competing interests

The authors declare date they have no conflict of interest.

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Authors' contributions

WS did the experiments with pulp bleaching and effluent treatment. HH, JE and SB designed the experiments and supervision the research. All authors read and approved the final manuscript.

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Abbreviations

BOD: Biochemical Oxygen Demand

Cp: Centipoise

CRD: Completely Randomized Design

FTIR: Fourier-Transform Infrared Spectroscopy

IENT: Industrial Effluent Standards Assigned in Thailand

kPa: Kilopascal

LSD: Least Significant Difference

mN: Millinewton

Nm/g: Newton Metre Per Gram

SEM: Scanning Electron Microscope

TDS: Total Dissolved Solids

TOC: Total Organic Carbon

TSS: Total Suspended Solids

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Figures

Figure 1

Effect of eucalyptus pulp bleaching treated by endo-1,4- β -xylanase A), untreated pulp; B), enzyme-treated pulp; C), chlorine dioxide-treated pulp. Features of surface pulp fiber are presented by arrow head.

Figure 2

FTIR spectra of eucalyptus pulp bleaching in D0 stage; A), untreated; B), enzyme treated and C), chemical treated.

Figure 3

FTIR spectra of effluent after eucalyptus pulp bleaching; A), untreated; B), endo-1,4- β -xylanase-treated and C), chlorine dioxide-treated.

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