EXPLORATION AND ANALYSIS OF A LIGNOCELLULASE CONSORTIUM FOR DEINKING OF RECYCLED PAPER IN THE ECO-PAPER INDUSTRY

Sylvia Aulia Rahmah[2], Nadya Aisya[1], Lailatul Fithri[2], Andre Pratama[1], Handoko Darmokoesoema[1], Ali Rohman[1,2], Ni Nyoman Tri Puspaningsih[1,2,3]

[1] *Department of Chemistry, Faculty of Science and Technology*
Universitas Airlangga, Kampus C-UNAIR
Mulyorejo Surabaya, East Java 60115, Indonesia
E-mail: ni-nyoman-t-p@fst.unair.ac.id
[2] *Proteomics Laboratory, Research Center for Bio-Molecule Engineering*
Universitas Airlangga, Kampus C-UNAIR, Mulyorejo Surabaya, East Java 60115, Indonesia
[3] *Department of Bioinformatics & Medical Engineering*
Asia University, Taiwan

Received 21 January 2019
Accepted 31 July 2019

ABSTRACT

Deinking is the process of removing and separating ink from printed paper fiber. Large quantities of chemicals are used in the traditional deinking process in the paper industry. This is not only expensive, but the use of chemicals in large quantities also leads to many environmental problems. To reduce the chemical usage, one eco-solution is to use enzymes as deinking agents. In this article, an enzymatic newspaper deinking using a lignocellulase cocktail consisting of a cellulase from Actinobacillus sp. and recombinant xylanase from Geobacillus thermoleovorans IT-08 expressed in Escherichia coli DH5α (namely pTP-510) is reported. In order to increase the brightness of the deinked paper, a laccase from the wild type fungus Aspergillus sp. was added to the enzyme cocktail. The ratio of cellulase to xylanase in the cocktail was 4:1 and the laccase activity was varied. To assess the deinked paper quality, its brightness, strength, and surface profile were analyzed. The results showed that the addition of laccase 0.291 U to the enzyme cocktail leads to a brightness level of 60.4 % (ISO 2470), which exceeds the SNI 14-0091-1998 standard of 55 % (ISO 2470). The condition for tensile index was 25.36 Nm/g (ISO 1924/2), which is greater than the SNI 14-0091-1998 standard of 21.5 Nm/g (ISO 1924/2). Moreover, scanning electron microscopy (SEM) revealed that the surface of the enzymatically deinked paper is more tenuous than the control.

Keywords: consortium lignocellulase, deinking recycling paper, brightness index, tensile index.

INTRODUCTION

Paper is a material used daily for many purposes worldwide, including newspapers, magazines, and photo copy paper. Generally, paper production uses wood as raw material and therefore, at least partly, causes deforestation. It is generally known that deforestation with the aim of harvesting wood is a major environmental problem. For example, deforestation is considered to be responsible for climate change [1]. To preserve the forest resources, non-wood raw materials are required for paper production.

One alternative method to produce paper is by recycling the used paper [2]. In the paper recycling process, the removal and the separation of ink from the printed paper fibers namely paper deinking, plays an important

role. In general, large quantities of chemicals are used during the traditional deinking process, which leads to other environmental problems. To reduce chemical usage in the deinking process, one eco-solution is using enzymes as deinking agents [3].

Lignocellulases are enzymes that consist of a large group of chiefly extracellular proteins including ligninolytic enzymes (oxidases and peroxidases) and hydrolytic enzymes (hemicellulases, cellulases, chitinases, pectinases, amylases, proteases, esterases, and mannases) [4]. Lignocellulases are applicable in biodegradation of synthetic dyes, including bio-bleaching of colored effluents from pulp, textile industries, paper, deinking, recycled paper, and in the decolorization of wastewater ([3, 5, 6]). The paper industry depends on many lignocellulose degrading enzymes and their capability in modifying paper pulp has been reported [7, 8]. Cellulase and xylanase are the most promising enzymes in paper and pulp modification [6].

Here the utilization of a lignocellulase consortium, composed of a cellulase and xylanase cocktail in the newspaper deinking process is reported. The cellulase was produced extracellularly by wild type *Actinobacillus* sp. and the recombinant xylanases were expressed in *E. coli* DH5α that harbors a pTP-510 plasmid. The plasmid carries a xylanolytic gene cluster from the thermophilic bacterium *Geobacillus thermoleovorans* IT-08. The gene cluster encodes the ß-D-xylosidase B (GbtXyl43B), ß-D-xylosidase A (GbtXyl43A), and α-L-arabinofuranosidase (Abfa) (GenBank Accession Nos. DQ387047, DQ345777, and DQ387046, respectively) [9]. To improve the brightness of the deinked paper, a fungal laccase produced by *Aspergillus* sp. was added to the enzyme cocktail.

EXPERIMENTAL

Microorganisms and culture

Cellulase and laccase were prepared from wild type *Actinobacillus* sp. and *Aspergillus* sp., respectively, while xylanase was produced by *E. coli* DH5α harboring a plasmid pTP-510 that contains a xylanolytic gene cluster from the bacterium *Geobacillus thermoleovorans* IT-08 [9]. All microorganisms are from the collection of the Proteomics Laboratory, Institute of Tropical Disease, Universitas Airlangga.

Cellulase

A stock culture of the bacterium *Actinobacillus* sp. was prepared on a Luria-Bertani (LB) agar medium supplemented with 1% (m v^{-1}) carboxyl methyl cellulose (CMC) and grown at 37°C for 18 h. The bacterial stock was stored at 4°C and sub-cultured every month. A pre-culture was grown from a single colony of the stock in 100 mL LB broth containing 1% (m v^{-1}) CMC by shaking at 150 rpm in a 37°C incubator overnight. Extracellular cellulase was produced by inoculating 1 L of the same medium with 1% (v v^{-1}) of the pre-culture and cells were grown under the same conditions as the pre-culture. After growing for 18 h, the cells were harvested by centrifugation at ~18000 *g* for 10 minutes at 4°C. Cellulase containing supernatant was collected and stored at 4°C until used. The enzyme was subsequently freeze-dried to test enzymatic activity [10].

Xylanase

A bacterial stock of *E. coli* DH5α harboring pTP-510 plasmid was streaked on LB agar containing 100 µg mL^{-1} ampicillin and stored at 4°C. A pre-culture from a colony of this *E. coli* stock was grown in 100 mL of LB broth supplemented with 100 µg mL^{-1} ampicillin by shaking at 150 rpm at 37°C overnight. The pre-culture was used to inoculate 1 L of fresh LB medium containing 100 µg mL^{-1} ampicillin and the culture was shaken at 150 rpm at 37°C for 3 h. At this point, xylanase expression was induced by supplementing the culture with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 h of induction, cells were harvested by centrifugation at ~18000 *g* for 10 minutes at 4°C. The cell pellet was resuspended in 30 mL of 100 mM phosphate-citrate (PC) buffer pH 7.0 and lysed by ultra-sonication. Cell debris was then separated by centrifugation at ~18000 *g* for 30 minutes at 4°C. The xylanase-containing supernatant was collected and stored at 4°C until used. The enzyme was subsequently freeze-dried to test enzymatic activity [9].

Laccase

The fungus *Aspergillus* sp. was grown on a potato dextrose agar (PDA) slant for 5 days at room temperature. The spores of this fungus were subsequently resuspended in sterile water and used for 1% (v v^{-1}) inoculation of 1 L glucose, yeast extract, and malt extract (GYM) broth. The culture was grown at room temperature with shaking at 150 rpm. When the fungal growth reached the log phase (*i.e.,* 3-day-old culture), $CuCl_2$ was added to the culture to a final concentration

of 0.5 mM. After growing for 7 days, the cells were harvested by centrifugation at ~18000 *g* for 10 minutes at 4°C. Cell-free supernatant was stored at 4°C and used for the laccase assay. The enzyme was subsequently freeze-dried to test enzymatic activity [11].

Enzymatic Assay

Cellulase

Cellulase activity was determined by measuring the amount of reducing sugar released from the CMC substrate by the enzyme [12]. The reducing sugar was measured by the DNS method [10]. The substrate was prepared by dissolving CMC in 100 mM PC buffer pH 6.0 to a concentration of 1% (m v^{-1}). In a typical assay, 100 µL of the substrate was mixed with 100 µL of the enzyme solution. After incubating the mixture for 60 minutes at 45°C, 600 µL of the DNS reagent was added, the mixture was boiled for 15 minutes, and then cooled on ice for 20 minutes. The optical density of the reaction mixture was then measured at 550 nm with a spectrophotometer. A control experiment was conducted by omitting the enzyme. The amount of the reducing sugar released for each reaction was calculated by plotting the optical density to a glucose calibration curve. The curve was prepared as in the enzyme assay experiments, by replacing substrate and enzyme solutions with 200 µL of glucose solution in 100 mM phosphate buffer at pH 6.0. The final glucose concentrations used to prepare the calibration curve ranged from 0.15 to 0.45 µg mL^{-1}. Cellulase activity (U mL^{-1}) was calculated using equation (1). One activity unit of cellulase corresponds to the amount of enzyme that hydrolyzes 1 µmol of glycosidic bonds of the CMC substrate per minute [13].

$$Cellulase\ activity\ \left(\frac{U}{ml}\right) = \frac{[Cx10xfp]}{T\ x\ MM\ glucose} \quad (1)$$

where C is the reducing sugar concentration, T is the incubation time, Fp is the dilution factor, and MM glucose is the molecular mass of glucose (180).

Xylanase

Xylanase activity was measured by a similar method used for the determination of cellulase activity described above [10], using oat spelt xylan as substrate. The substrate was dissolved in 100 mM PC buffer pH 7.0 to a concentration of 1% (m v^{-1}). Typically, a mixture of 100 µL of the substrate solution and 100 µL of the enzyme solution was incubated at 70°C for 60 minutes. The reaction was stopped by adding 600 µL of the DNS reagent and boiling for 15 minutes. After cooling on ice for 20 minutes, the optical density of the reaction mixture was measured at 550 nm using a spectrophotometer. A blank assay was performed by replacing the enzyme solution with 100 mM PC buffer pH 7.0. The concentration of the reducing sugar released for each reaction was calculated based on a xylose calibration curve. This curve was prepared by a method similar to the above-mentioned glucose calibration curve, using xylose instead of glucose, with final concentrations ranging from 0.3 to 10 mg mL^{-1}.

Xylanase activity (U mL^{-1}) was calculated in equation (2). One activity unit of xylanase corresponds to the quantity of the enzyme that hydrolyzes 1 µmol of glycosidic bonds of the oat spelt xylan substrate per minute [13].

$$Xylanase\ activity\ \left(\frac{U}{ml}\right) = \frac{[Cx10xfp]}{T\ x\ MM\ glucose} \quad (2)$$

where C is the reducing sugar concentration, T is the incubation time, Fp is the dilution factor, and MM glucose is the molecular mass of glucose (180).

Laccase

Laccase activity was determined by the 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method. In this method, the non-phenolic dye ABTS is oxidized by laccase to a cationic radical. The concentration of the cationic radical, which absorbs light at a wavelength of 420 nm, corresponds to laccase activity [14]. The assay mixture contained 1160 µL of 0.4 mM ABTS in PC buffer pH 7 and 40 µL of the enzyme solution. This mixture was incubated at 50°C for 30 minutes and its optical density was measured at 420 nm every 5 minutes against a control [14]. The control was prepared by replacing the enzyme solution with the assay buffer. One unit of laccase activity, which was calculated using equation (3), was defined as the amount of the laccase that oxidizes 1 μmol of the ABTS substrate per minute. Laccase activity (U mL^{-1}) can be calculated by the formula:

$$Activity\ \left(\frac{IU}{l}\right) = 2\left(\frac{V}{vx\varepsilon xd}\right) x\Delta A.min^{-1}x1000 \quad (3)$$

where V is the total reaction volume in mL, ν is the enzyme volume in mL, ε is the extinction coefficient

ABTS at 420 nm (36 mM^{-1} cm^{-1}), *d* is the light path of cuvette in cm, and $\Delta A.min^{-1}$ is the change of absorbance every 5 min at 420 nm.

Paper Biodeinking

The first process in this treatment was making a pulp. Recycled newspaper stock was made by adding 150 grams of recycled newspaper and water to a total of 1850 grams for a consistency of ~7.5 %. Then the mixture was added into mini pulper with 1500 scale and repeated up to five times. 600 grams of homogeneous pulp was added for bio-deinking.

Three types of lignocellulases were used for bio-deinking process. The first - only used xylanase with activity variation 2.2055; 4.4110; 6.6165 U and incubation at 70°C for 30 minutes; the second - combination of cellulase and xylanase with activity ratio 3:1 without laccase; the third - combination of cellulase and xylanase with addition of laccase. Dose of cellulase and xylanase was 650 ppm with activity ratio 4:1. Then laccase was added with activity ratio of 0.036 : 0.072 : 0.109 : 0.145 : 0.291 : 0.436 : 0.582 U. This variation aims to determine the optimum ratio of laccase for the same substrate level.

Bio-deinking process for combination of cellulose, xylanase, and laccase used of 3 different temperatures. Incubation with cellulase was 45°C for 15 minutes, followed by incubation with laccase at 50°C for 15 minutes. The final step was incubated with xylanase at 70°C for 15 minutes. The 0.15 % of deinking agent (surfactant) was added and stirred for 1 minute.

The next process was flotation, and the biodeinked pulp was run at a consistency of 0.9 % for 5 minutes to remove the ink released from the paper fibers. The deinked pulp was made into a sheet of 55 g m^{-2}.

Physical Analysis

The deinked paper pulp was analyzed for brightness and tensile strength, and its surface profile was visualized by scanning electron microscopy (SEM).

RESULTS AND DISCUSSION

Enzymatic Assay

The assay results indicate that cellulase activity before and after freeze-drying was 0.0675 U mL^{-1} and 0.1425 U mL^{-1}, respectively. The xylanase activity assay results before and after freeze-drying were 0.1267 U mL^{-1} and 0.1295 U mL^{-1}, respectively. Changes in absorbance for every 5 minutes of laccase addition gave a linear graph curve and the equation y = 0.00042x + 0.173. Therefore, laccase activity before and after freeze-drying was 0.0175 U mL^{-1} and 0.1245 U mL^{-1}, respectively.

Physical Analysis

Brightness

Indonesian National Standard (Abbreviated SNI) is the only standards that apply nationally in Indonesia. According to SNI 14-0091-1998, standard of paper brightness value, is 55 %.

The first and second assay showed that the addition of xylanase and cellulase (step 1 and step 2) for deinking pulp indicated less than the Indonesian Standard of SNI (Fig. 1, Fig. 2). However, the third assay after laccase addition to the enzyme cocktail improved the paper brightness above the SNI. The brightness was increased in conjunction with increasing the laccase activity. The optimum value of brightness was ~60.4 % when 0.291 U of laccase was added to the cocktail (Fig. 3).

It is possible that the addition of laccase in the cocktail facilitates degradation of lignin that constitutes the paper sample and therefore makes the resulting paper brighter [15, 16]. The best paper brightness value reported here is in accordance with the value required by the Indonesian Standard of SNI 14-0091-1998 of 55 % [17]. Moreover, this value is higher than the previously reported value of 54.11% for paper deinking using cellulase and xylanase cocktail [18].

Tensile Strength

The paper tensile strength is the maximum tension required to break a strip of paper sheet [19]. Treatment of paper pulp with the first, second and third assay was in accordance with the value required by the Indonesian Standard of SNI (Fig. 4, Fig. 5, Fig. 6). The best treatment of paper pulp was with laccase (third assay). During the deinking process, addition of laccase decreased the tensile strength of the paper with value ~25.36 Nm g^{-1} when laccase of ~0.145 U or higher was applied (Fig. 6). As mentioned above, laccase may facilitate lignin degradation [16, 20]. This third assay revealed that the best value of tensile strength better than first and second assay, respectively.

The tensile strength result showed that the addition of laccase to the mixture of cellulase and xylanase for deinking decreased the tensile strength. The analysis

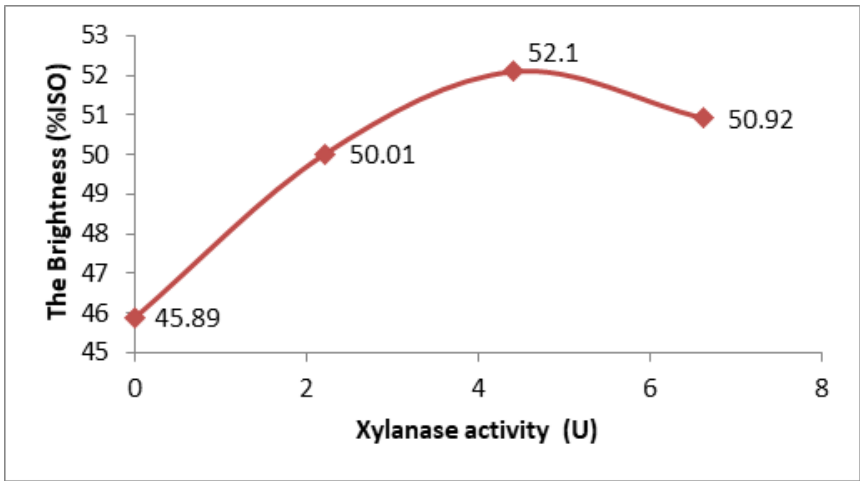

Fig. 1. A relationship between xylanase activity and brightness.

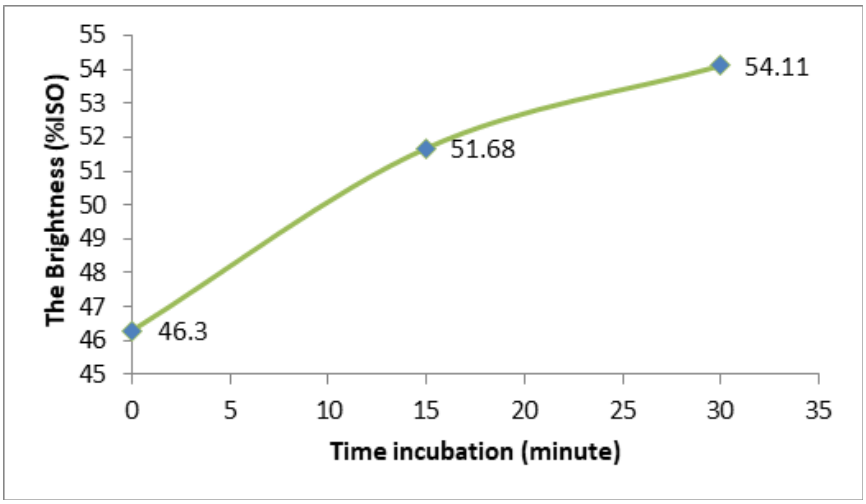

Fig. 2. A relationship between the time variation of cocktail enzymes without laccase and brightness.

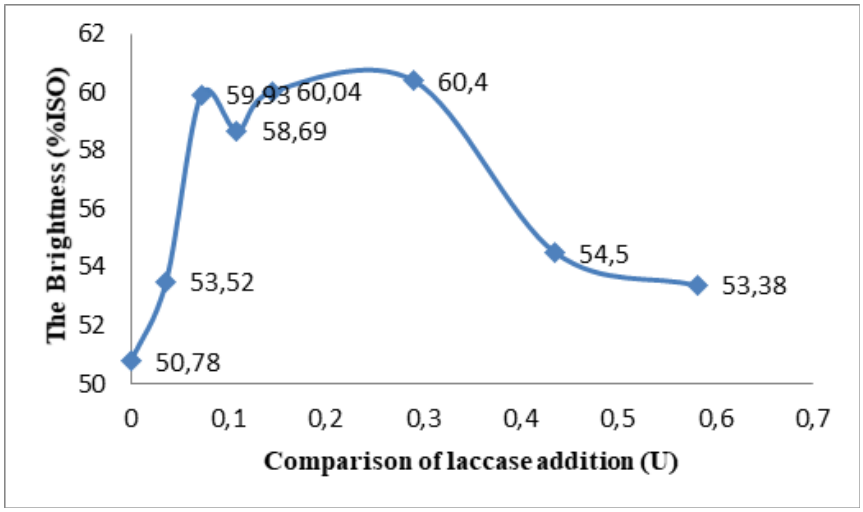

Fig. 3. A relationship between the laccase addition and brightness.

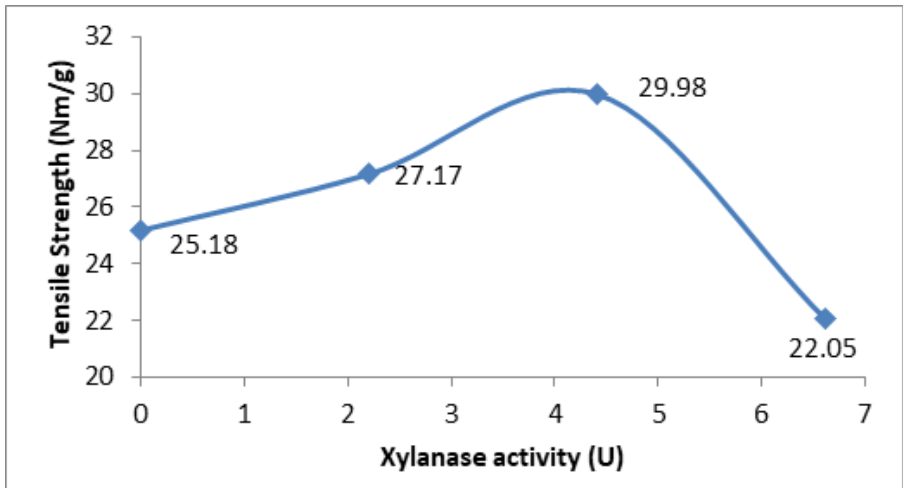

Fig. 4. A relationship between the xylanase activity and tensile strength.

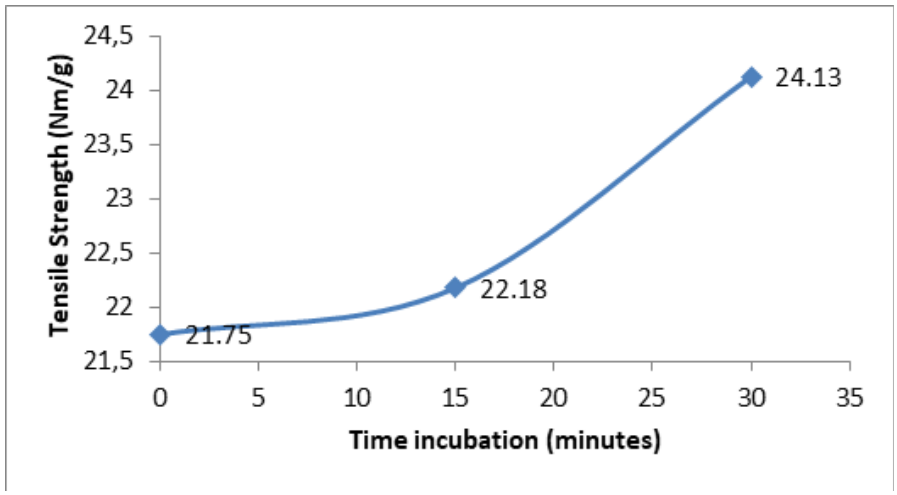

Fig. 5. A relationship between the time variation of cocktail enzymes without laccase and tensile strength.

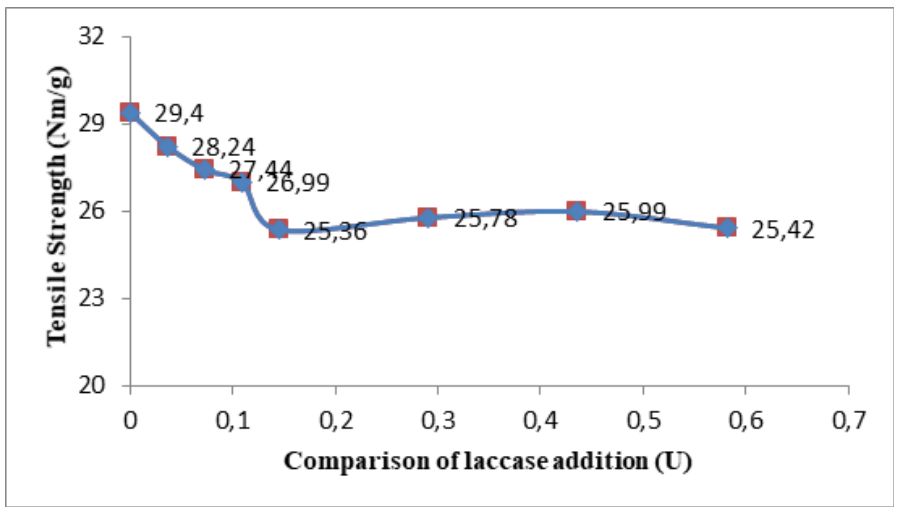

Fig. 6. A relationship between the laccase addition and tensile strength.

was done using autograph AG-10 Shimadzu. It may suggest that laccase can remove the lignin binding to the fibers on the paper [20]. In general, laccase converts the lignin into a less-damaging product, using electron transfer and hydrogen atom transfer mediators [15]. Lignin is the most abundant organic compound after cellulose and is a large compound made up of phenyl-propane units linked in a three-dimensional structure [21, 22]. Cellulose microfibrils are attached to each other by hemicellulose and/or pectin and held together by lignin. Diverge chains of hemicelluloses support and build a network with cellulose microfibrils and interact with lignin, making the cellulose-hemicellulose-lignin matrix extremely strong. Lignin plays the role of cement by cross-linking cellulose and hemicellulose to form a rigid three-dimensional structure of the cell wall [20, 22]. Therefore, if the lignin breaks down due to addition of laccase, which helps weaken the binding between cellulose and xylanase, the deinked pulp will have decreased tensile strength [20].

The minimum tensile strength level was at 0.145 U laccase addition. Laccase activity and the deinked pulp meets the SNI 14-0091-1998 standard in this case.

SEM (Scanning Electron Microscopy)

SEM analysis showed that there was a modification in the surface structure following enzymatic treatment, which can be seen in Fig. 7. The sample for SEM was deinked pulp with 0.291 U of added laccase, and the control was pulp without enzyme addition. Fig. 3 shows that laccase addition to the mixture of cellulase and xylanase made deinked pulp weaker. More fibrillation could be observed for laccase-deinked pulp. Some hollows, probably resulting from the treatment, were present. It is possible that the enzymes removed lignin binding from the fibers on the paper.

The results correlate with a previous study on the effects of adding a combined laccase-mediator system with hemicellulase to newsprint pulp [23]. Defibrillations, crack formations, and changes in functional groups were also observed from deinked paper pulp treatment by the cellulase-xylanase complex from *E. coli* SD5 [6].

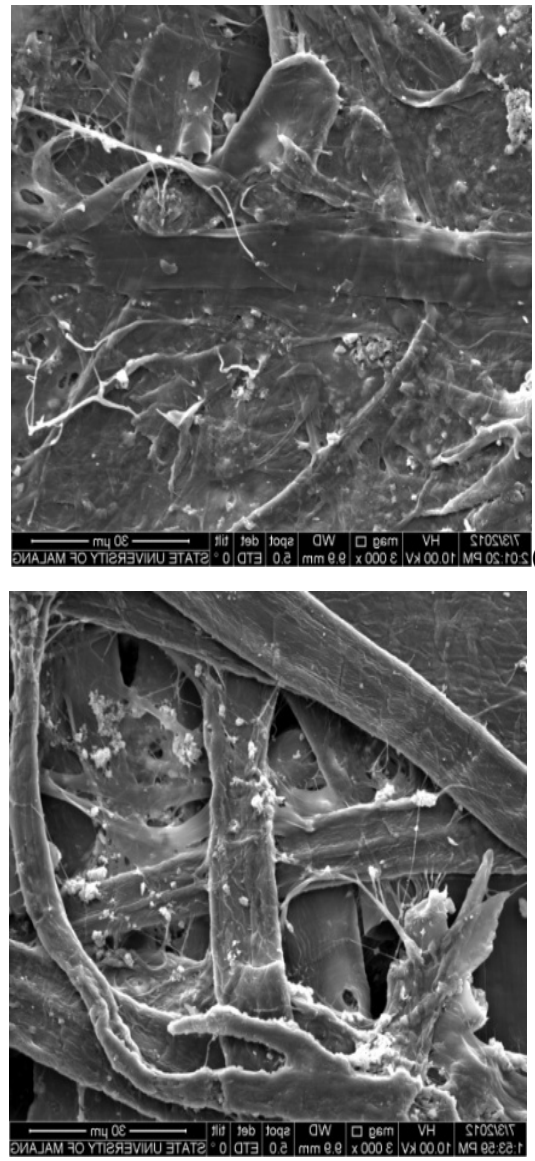

Fig. 7. Scanning electron micrographs of deinked pulp (a) control without addition enzymes (3000x); (b) sample with addition of laccase activity at 0.291 U (3000x).

CONCLUSIONS

The application of an enzyme cocktail containing cellulase, xylanase and laccase in the paper deinking process improves brightness of the deinked paper, but reduces its strength. However, the strength of the resulting deinked paper still meets the standard of SNI 14-0091-1998.

Acknowledgements

The authors would like to thank the Ministry of Research, Technology and Higher Education for research funding, PT. Adiprima Suraprinta (Paper Industry) for supporting this research.

REFERENCES

1. J. Hein, A. Guarin, E. Frommé, P. Pauw, Deforestation and the Paris climate Agreement: An Assessment of REDD + in the national climate action plans, Forest. Policy. Econ, 90, 2018, 7-11.
2. I. Ervasti, R. Miranda, I. Kauranen, Paper recycling framework, the "Wheel of Fiber", J. Environ. Manage, 174, 2016, 35-44.
3. D.E. Tsatsis, D.K. Papachritos, A.G. Valta, A.G. Vlyssides, D.G. Economides, Enzymatic deinking for recycling of office waste paper, J. Environ. Chem. Eng, 5, 2, 2017, 1744-1753.
4. G.Y.S. Mtui, Lignocellulytic enzymes from tropical fungi: Types, substrates and applications, Sci. Res. Essays, 7, 15, 2012, 1544-1555.
5. W. Apiwatanapiwat, P. Siriacha, P. Vaithanomsat, Screening of Fungi for Decolorization of Wastewater from Pulp and Paper Industry, Kasetsart J. (Nat. Sci), 40, 2006, 215-221.
6. N.V. Kumar, M.E. Rani, R. Gunaseeli, N.D. Kannan, Paper Pulp Modification and Deinking Efficiency of Cellulase-Xylanase Complex from Escherichia coli SD5, Int. J. Biol. Macromol, 111, 2018, 289-295.
7. A. Saxena, P. Singh Chauhan, Role of Various Enzymes for Deinking Paper: a Review, Crit. Rev. Biotechnol, 37, 5, 2017, 598-612.
8. V.K. Nathan, M.E. Rani, G. Rathinasamy, K.N. Dhiraviam, S. Jayavel, Process optimization and production kinetics for cellulase production by Trichoderma viride VKF3, Springer Plus, 3, 2014, 92.
9. N.N.T Puspaningsih, Gene Encoding Xylosidase from Bacillus thermoleovorans IT-08 (Gen Penyandi Xilosidase dari Bacillus thermoleovorans IT-08), Dissertation, Bogor Agricultural University (IPB), 2004.
10. G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem, 31, 3, 1959, 426-428.
11. E.C. Stevenson, T. Park, J.W. Mitchell, Method of Culturing Fungi, United States Patent Office, Patent Mar.16, 1948, US2437766A, https://patents.google.com/patent/US2390941
12. T. Shuangqi, W. Zhenyu, F. Ziluan, Z. Lili, W. Jichang, Determination methods of cellulase activity, Afr. J. Biotechnol, 10, 37, 2011, 7122-7125.
13. A.V. Gusakov, E.G. Kondratyeva, A.P. Sinitsyn, Comparison of Two Methods for Assaying Reducing Sugars in the Determination of Carbohydrase Activities, Int. J. Anal. Chem, 2011, 1-4.
14. R. Bourbonnais, D. Leech, M.G. Paice, Electrochemical analysis of the interactions of laccase mediators with lignin model compounds, Biochimia et Biophysica Acta (BBA) - General Subjects, 3, 1998, 381-390.
15. E.M. Obeng, S.N.N. Adam, C. Budiman, C. M. Ongkudon, R. Maas, J. Jose, Lignocellulases: a review of emerging and developing enzymes, systems, and practices, Bioresour. Bioprocess, 4, 2017, 16.
16. Q. Wang, M.S. Jahan, S. Liu, Q. Miao, Y. Ni, Lignin Removal Enhancement from Prehydrolysis Liquor of Kraft-based Dissolving Pulp Production by Laccase-induced Polymerization, Bioresour. Technol, 164, 2014, 380-385.
17. Indonesian National Standard for News Paper (SNI, Standar Nasional Indonesia), SNI 14-0091-1998.
18. W. Pilarsih, Brightness Enhancement on Newsprint Deinking Process by a Combination of Cellulase Enzyme and Recombinant Xylanolitic Enzyme, Report prepared for final examination undergraduate student, Department of Chemistry Universitas Airlangga, 2012.
19. D. Muchorski, Tensile properties of paper and paperboard (using constant rate of elongation apparatus), TAPPI, 2006, http://www.tappi.org/content/SARG/T494.pdf.
20. X. Lin, Z. Wu, C. Zhang, S. Liu, S. Nie, Enzymatic Pulping of Lignocellulosic Biomass, Ind. Crop. Prod, 120, 2018, 16-24.
21. E.H. Koupaie, S. Dahadha, A.A. Bazyar Lakeh, A. Azizi, E. Elbeshbishyi, Enzymatic pretreatment of lignocellulosic biomass for enhanced biomethane production-A review, J. Environ. Manage, 2018, https://doi.org/10.1016/j.jenvman.2018.09.106.
22. Y. Zheng, J. Zhao, F. Xu, Y. Li, Pretreatment of lignocellulosic biomass for enhanced biogas production, Prog. Energ. Combust. Sci, 42, 2014, 35-53.
23. Q.H. Xu, Y.P. Wang, M.H. Qin, Y.J. Fu, Z.Q. Li, F.S Zhang, J.H. Li, Fiber surface characterization of old newsprint pulp deinked by combining hemicellulase with laccase-mediator system, Bioresour. Technol, 102, 11, 2011, 6536-6540.