A Pronounced Improvement of the Kappa Number Reduction and Pulp Properties Associated with the Use of Extracellular Enzymes Secreted by Selected Fungal Strains

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A combination of extracellular enzymes secreted by *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 showed promising results in the reduction of kappa number of *Acacia* oxygen-delignified kraft pulp (A-OKP) in previous studies. However, the observed Kappa number reduction was low, and the bleaching process required further optimization. In the current study, the A-OKP was treated with a combination of extracellular enzymes of *I. lacteus* and *L. tigrinus*, with a subsequent alkaline peroxide extraction, which significantly improved the Kappa number reduction. The maximum achieved Kappa number reduction was 26%. The effects of static incubation and sterilization of the extracellular enzymes on biobleaching process were evaluated. Compared with a static biobleaching, biobleaching with shaking shortened the required incubation time required from 3 to 1 d. The utility of extracellular enzymes was tested with and without sterilization; no significant differences in Kappa number reduction, brightness, and physical properties of the pulp were observed. The physical properties of all pulp samples were improved following the enzymatic treatment. Furthermore, a low-cost medium containing wood powder supplemented with rice bran and palm sugar (WRBP) was used for the production of enzymes for biobleaching of A-OKP.

Keywords: Acacia kraft pulp; Biobleaching; Kappa number; Peroxide extraction

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INTRODUCTION

The use of conventional chlorine-based chemicals in bleaching sequences at kraft mills is currently being reduced because of environmental concerns. As a new environmentally benign bleaching method, the use of enzymes has made an impact on the industrial bleaching of kraft pulp (Farrell *et al*. 1996). Most research to reduce the amount of chlorine in the pulp and paper industry has recently focused on hemicellulase, *e.g.*, xylanase (Sharma and Kumar 2013); however, oxidative enzymes from white-rot fungi have been successfully applied in biobleaching on a laboratory scale (Moreira *et al*. 2003; Ibarra *et al*. 2006). The potential of incorporating oxidative enzymes for the development of chlorine-free pulp bleaching processes has been intensively studied (Paice *et al*. 1995). The oxidative enzymes secreted by white-rot fungi include manganese-dependent peroxidase (MnP) (Glenn and Gold 1985), lignin peroxidase (LiP) (Tien and Kirk 1984), manganese-independent peroxidase (MIP) (de Jong *et al*. 1992), and laccase (Lac) (Youn *et al*. 1995). Studies with different white-rot fungi revealed that MnP is more commonly

correlated with delignification during biobleaching than LiP or Lac (Orth *et al.* 1993); the characteristics and role of MnP in biobleaching process have been extensively studied (Wariishi *et al.* 1991; Palma *et al.* 2000; Tello *et al.* 2000).

Despite extensive investigations of oxidative enzymes from white-rot fungi for biobleaching, it has yet to be industrialized. The major challenges in using of oxidative enzymes for pulp bleaching are cost and environmental health. For example, Lac is the most studied for biobleaching, requiring molecular oxygen and mediators such as 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT) (Call and Mücke 1997; Eugenio *et al*. 2010); however, utilization of ABTS or HBT as mediator, in industrial processes is hindered by their high cost and toxicity (Eugenio *et al*. 2010). In addition, using purified MnP in the mill is costly because this enzyme requires organic acid to stabilize Mn^{3+} and hydrogen peroxide for electron acceptor (Moreira *et al.* 2001).

The idea of this study is to find a system in biobleaching in which the enzymes are able to act like they do in their natural situation. Bajpai *et al.* (2006) have reported that the delignification and brightening observed with the fungus are not only due to MnP, LiP, or Lac alone, but also to the presence of some enzymes and cofactors in the system. This is because a single enzyme is not able to mimic the complete biological system. In addition, degradation of wood components in nature is a complex process involving the synergistic action of a large number of extracellular enzymes, together with low molecular-weight cofactors (Leonowicz *et al*. 1999; Aro *et al*. 2005). All these enzymes cooperate to degrade wood component in nature. This synergistic action of the enzymes could be imitated in biobleaching.

Recently, we reported that extracellular enzymes secreted by *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 have shown promising results in biobleaching of *Acacia* oxygen-delignified kraft pulp (A-OKP) using low-cost media of agricultural and waste forestry (Afrida *et al.* 2014). During biobleaching with the culture media, there was no addition of mediators or other factors. These results clearly show that the *I. lacteus* and *L. tigrinus* secreted some compounds into culture media that might stabilize the extracellular enzymes during biobleaching. However, some stages of the biobleaching process should be further improved to optimize Kappa number reduction, brightness, and physical properties of the pulp after enzymatic treatment.

The objective of the present study was to examine the biobleaching process by combining a treatment with extracellular enzymes produced by *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 and a subsequent alkaline peroxide extraction. The biobleaching activities of these fungi greatly exceeded those of the highly lignin-degradative fungi *Phanerochaete chrysosporium* and *Trametes versicolor* (Afrida *et al.* 2009). The fungi were grown in a low-cost medium containing agricultural and forestry waste of wood rice bran palm sugar for the biobleaching of A-OKP.

EXPERIMENTAL

Materials

Fungal cultures

Irpex lacteus KB-1.1 and *Lentinus tigrinus* LP-7 were maintained on agar slants at 30 °C. The agar medium was prepared using 0.2% *Acacia mangium* wood powder, 0.01%

guaiacol, and 1.6% potato dextrose agar (PDA) (Nishida *et al*. 1988). The pre-inocula were obtained by incubating the fungi on PDA at 30 °C for 7 d.

Culture media and enzyme production

The time-course determinations of lignolytic enzyme production were carried out in shallow static liquid cultures (10 mL media in 100 mL Erlenmeyer flasks) at 30 °C containing *A. mangium* wood powder (40- to 60-mesh) and rice bran (3:2) in a 1% aqueous solution of palm sugar (WRBP). The flasks were inoculated with single plugs (6 mm in diameter) from 1-week-old PDA cultures and incubated without shaking at 30 °C. Every 3 d (for 21 d), the cultures were filtered and centrifuged at 10,000 rpm (r = 55 mm), at 4 °C for 10 min, and the supernatants were used to assay enzyme activity. The experiment was carried out in triplicate.

Methods

Enzyme activity assays

Enzyme activities were determined using a UV-1600 Shimadzu spectrophotometer (Kyoto, Japan) at 40 °C. The method of Hirai *et al*. (1994) was slightly modified to determine lignolytic activity. MnP, MIP, and Lac activities were determined by monitoring the oxidation of 2,6-dimethoxyphenol at A_{470}. The reaction mixtures for MnP activity contained 1 mM 2,6-dimethoxyphenol, 0.2 mM H_2O_2, and 1 mM $MnSO_4$ in 50 mM malonate buffer (pH 4.5). The reaction mixtures for MIP activity were the same as for MnP, except that 1 mM EDTA was used instead of 1 mM $MnSO_4$. The reaction mixtures for Lac activity contained 1 mM 2,6-dimethoxyphenol in 50 mM malonate buffer (pH 4.5). LiP activity was determined by monitoring the oxidation of veratryl alcohol at A_{310}; reaction mixtures contained 1.7 mM veratryl alcohol and 0.2 mM H_2O_2 in 20 mM succinate buffer (pH 3.0).

Cellulase activity determination

The cellulase activity content of the pooled extracellular enzyme fractions of *I. lacteus* KB-1.1 and *L. tigrinus* LP-7, which was used for biobleaching treatment, was examined. The cellulase activity was determined by endo-β-1,4-glucanase activity by measuring the amount of reducing sugars released from low-viscosity carboxymethylcellulose (2% w/v) in 50 mM sodium citrate buffer (pH 4.8) at 40 °C for 30 min (Ghose 1987). A glucose standard curve was used to calculate the cellulase activity. The release of reducing sugars was measured using the dinitrosalicylic acid method (Miller 1959).

Pulp treatment

The industrial A-OKP was characterized by the International Standard Organization (ISO) brightness of 47.6% and Kappa number of 9. To determine the effect of consistency on biobleaching process of A-OKP, extracellular enzymes from *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 cultures grown in WRBP medium were combined. The cultures were carried out in 100 mL Erlenmeyer flasks, with A-OKP that had been washed with distilled water until the filtrate was colorless. Different volumes of extracellular enzymes from WRBP cultures with maximum MnP activity were added to the A-OKP samples (equivalent to 1 g oven-dried weight), as follows: 10 mL (10% pulp consistency), 20 mL (5%), 34 mL (3%), and 50 mL (2%). The flasks were incubated statically at 40 °C for 3 d. The enzyme-free controls were performed in parallel and processed in the same

manner as the enzyme-treated pulp. After treatment, the pulp samples were filtered and washed with distilled water, with a subsequent alkaline peroxide extraction. This experiment was carried out in triplicate and in parallel.

The effect of static incubation on the rate of the biobleaching process was determined as follows. The washed A-OKP was sterilized (121 °C, 20 min) in 200 mL Erlenmeyer flasks. A-OKP samples (equivalent to 6 g oven-dried weight) were treated with 120 mL of combined extracellular enzymes of *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 (from WRBP cultures with a maximum MnP activity) and statically incubated at 40 °C for 3 d. In the controls, the extracellular enzymes were replaced with distilled water. After treatment, the pulp samples were filtered and washed with distilled water, with subsequent alkaline peroxide extraction. Two replicate enzymatic and control treatments were performed.

The effect of sterilizing the extracellular enzymes on the time-efficiency of the biobleaching process was assessed as follows. The extracellular enzymes were collected from *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 cultures. For the "sterile" biobleaching condition, extracellular enzymes were prepared by filtration through a sterile Millipore filter (0.22 μm). For the "non-sterile" condition, the extracellular enzymes were used without filtration. The combined extracellular enzymes solutions (120 mL) were added to the A-OKP samples (equivalent to 6 g oven-dried weight) that had been sterilized beforehand by autoclaving (121 °C, 20 min). The flasks were incubated horizontally, with shaking (150 rpm) at 40 °C for 24 h. After the treatment, the pulp samples were filtered and washed with distilled water, with a subsequent alkaline peroxide extraction; their physical properties were then analyzed. Two replicate enzymatic and control treatments were performed.

Alkaline peroxide extraction

A modified alkaline peroxide extraction method of Wong *et al.* (1999) was used. The chelation step was carried out in double plastic bags with 3% pulp consistency and 1% Na-EDTA at 50 °C for 1 h. After the pulp was washed with distilled water, the extraction was carried out with 10% pulp consistency, 0.5% $MgSO_4$, 2% Na_2SiO_3, 2% NaOH, and 2% peroxide at 80 °C for 2 h. After treatment, the pulp was filtered and washed with hot distilled water (60 to 70 °C).

Analytical techniques

All measurements were made according to the guidelines of Technical Association of the Pulp and Paper Industry (TAPPI), except for viscosity measurements, which were performed according to the Japan Wood Research Society (JWRS 2000). Pulp brightness was determined by using a colorimeter (Suga Test Instruments Co., Ltd, Tokyo, Japan). In this method, the brightness of white, near-white, and natural-colored pulp and paper is determined based on the directional reflectance factor at 457 nm (TAPPI T 452 om-92 1996). The Kappa number was determined using a micro-Kappa number measurement method (Berzins 1966). This micro-Kappa number can be used for the determination of the degree of pulp delignification and is equal to the volume (in mL) of 0.1 N potassium permanganate solutions that is consumed by 1 g of a moisture-free pulp under the appropriate conditions (TAPPI T 236 cm-85 1996). Laboratory handsheets were prepared (grammage of 60 g/m^2) for the determination of the physical properties of the pulp (TAPPI TAPPI T 205 sp-95 1996), namely thickness (TAPPI T 411 om-89 1996), burst index

(TAPPI T 403 om-91 1996), tensile index (TAPPI T 494 om-88 1996), and tear index (TAPPI T 414 om-88 1996).

RESULTS AND DISCUSSION

Extracellular Lignolytic Enzyme Activities

The time-course of lignolytic enzyme production by *I. lacteus* and *L. tigrinus* in shallow static culture in the economical WRBP medium was analyzed. Figure 1 shows the lignolytic activities of both strains grown in the WRBP medium.

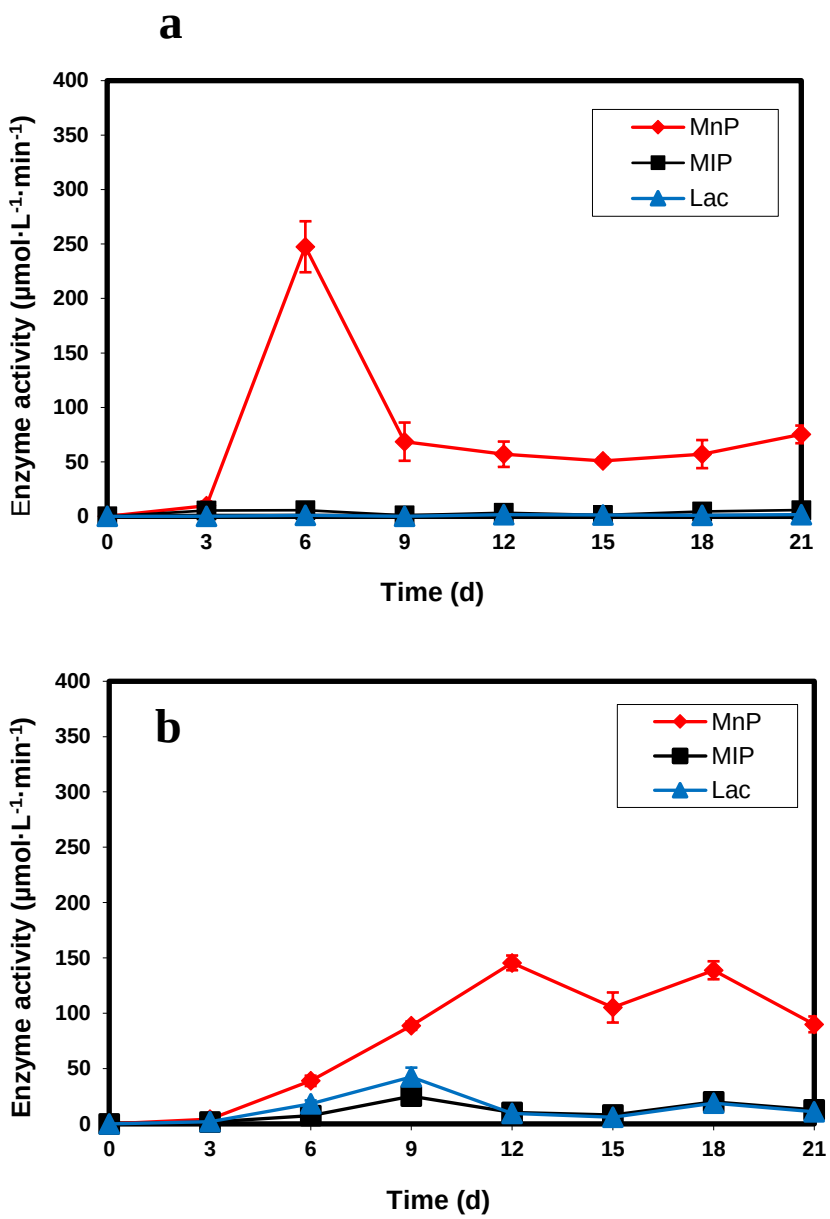

Fig. 1. Time-courses of lignolytic enzyme production *I. lacteus* KB-1.1 (a) and *L. tigrinus* LP-7 (b). Filled diamond, MnP; filled square, MIP; filled triangle, Lac

Both strains produced MnP, MIP, and Lac; the MnP activity was the highest among these lignolytic enzymes. The lignolytic enzyme activities were higher in *L. tigrinus* cultures than in *I. lacteus* cultures; the only exception was the peak MnP activity in *I. lacteus* culture, which was 2-fold higher than that in the *L. tigrinus* culture. A maximum production of MnP was observed on days 6 and 12 for *I. lacteus* and *L. tigrinus*, respectively. These results indicated that neither *I. lacteus* nor *L. tigrinus* excreted LiP into the medium. The maximum enzyme activities obtained were as follows: MnP, 74 ± 7.5 $\mu mol \cdot L^{-1} \cdot min^{-1}$; MIP 41 ± 4.0 $\mu mol \cdot L^{-1} \cdot min^{-1}$; and Lac, 18 ± 4.7 $\mu mol \cdot L^{-1} \cdot min^{-1}$.

In the current study, the use of WRBP medium leads to higher lignolytic activities in *I. lacteus* and *L. tigrinus* cultures, *ca*. 1 to 5 fold, than when WRBG medium is used (Afrida *et al*. 2014). Both strains produced MnP, MIP, and Lac, but not LiP. Previous studies show that *I. lacteus* produces LiP in shallow stationary culture under nitrogen-rich conditions (Novotný *et al*. 2004) and in a non-immersed liquid culture under nitrogen limitation (Rothschild *et al*. 2002). Thus, the type of substrate in the culture medium appears to determine the type and amount of extracellular enzymes produced by *I. lacteus*. There are no published reports on the ability of *L. tigrinus* to secrete LiP into the culture medium.

The growth of fungi was accompanied by the acidification of WRBP culture, with the pH approaching 5 after 21 days (Fig. 2). The rapid acidification of WRBP culture during the growth of *I. lacteus* and *L. tigrinus* was similar to that seen in the WRBG culture, with the pH reaching a value close to 5 at 21 d (Afrida *et al*. 2014).

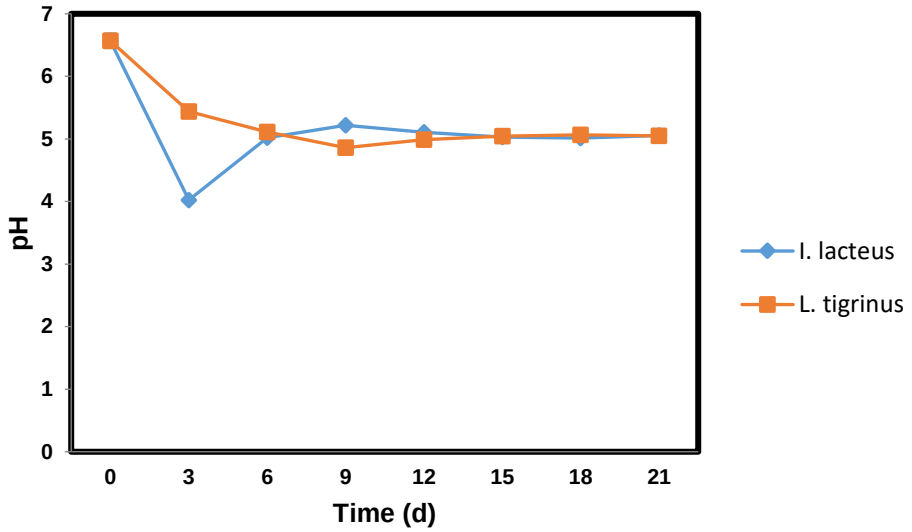

Fig. 2. Change in culture pH of *I. lacteus* KB-1.1 (filled diamond) and *L. tigrinus* LP-7 (filled square) culture during lignolytic enzyme production

Pulp Treatment

Pooling of the extracellular enzymes of *I. lacteus* and *L. tigrinus* results in a greater Kappa number reduction (7.4%) than when single extracellular enzyme fractions are used (*I. lacteus*, 4.4% and *L. tigrinus*, 6.7%) (Afrida *et al*. 2014). Similarly, Kumar and Kumar (2004) observed that a combination of several bacteria for biobleaching is more effective than the use of a single bacterium. Moreover, a combination of fungal species has been used for the bioremediation of pulp and paper mill effluent (Malaviya and Rathore 2007).

The pooling of several bacteria or fungi, or extracellular enzymes, gives better results than the use of a single bacterium of fungus, or any single extracellular enzyme. Considering of these results, in the present work a combination of the extracellular enzymes of *I. lacteus* and *L. tigrinus* was employed with the maximum MnP for the biobleaching. MnP is a key enzyme in the fungal biobleaching of kraft pulp (Bajpai *et al*. 2006). Moreira *et al.* (2001) reported that the maximum brightness obtained and a decrease in Kappa number of eucalyptus oxygen-delignified kraft pulp (ODKP) associated mostly to the production of MnP. For this reason, we used the MnP maximum for collecting the extracellular enzymes for biobleaching of A-OKP.

The feasibility of using fractions of *I. lacteus* and *L. tigrinus* WRBP cultures with the maximum MnP activity for the biobleaching of A-OKP was next examined. The effect of pulp consistency on the ability of the extracellular enzymes from WRBP culture to A-OKP was investigated using pulp preparations of four different consistencies (2%, 3%, 5%, and 10%) and was further examined with peroxide extraction to Kappa number reduction (Fig. 3).

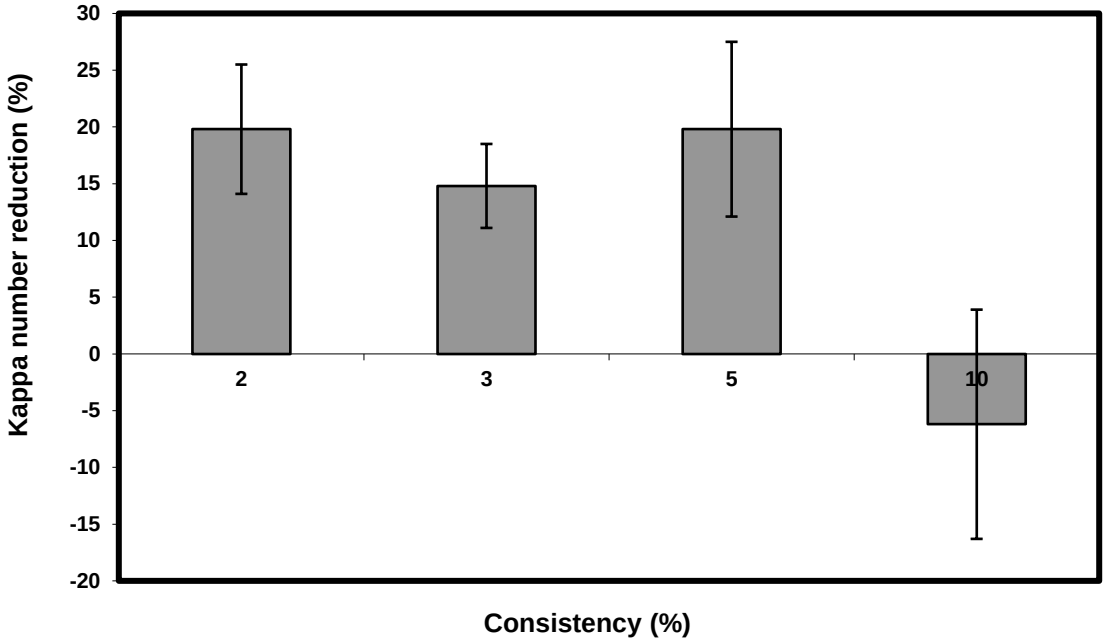

Fig. 3. The effect of pulp consistency on kappa number reduction of A-OKP samples upon treatment with the combined extracellular enzymes from *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 WRBP medium cultures

The results indicated the importance of the pulp-to-extracellular ratio. When the consistency was 2%, 3%, and 5%, the Kappa number reduction was remarkably decreased (19.8%, 14.8%, and 19.8%, respectively). The effects of these three pulp consistencies on the Kappa number reduction were not significantly different, as assessed by one-way ANOVA ($P > 0.05$). However, when the consistency was further increased from 5% to 10%, the Kappa number reduction was smaller, by about 6.2% ($P < 0.05$). Considering these results, 5% consistency was used in subsequent experiment. Geng *et al*. (2004) found that during softwood kraft pulp bleaching with a Lac/N-(-4-cyanophenyl)acetohydroxamic acid system, 5% pulp consistency resulted in a greater in Kappa number reduction than 10% pulp consistency. The optimal pulp consistency for decreasing the Kappa number in

the current study was in the range of 2 to 5%, which coincides with the pulp consistency employed in other experiments (Wong *et al*. 1999; Ibarra *et al*. 2006; Fillat *et al*. 2010). Presumably, low pulp consistency facilitates the mixing and dissolution of enzymes into the pulp. Moreover, low consistency can facilitate the diffusion of lignolytic enzymes into pulp fibers.

Next, the effect of the incubation mode (static or shaking) on the biobleaching process was determined. There was a change of the pulp color during A-OKP biobleaching with extracellular enzymes (decolorization). The incubation was discontinued when the pulp changed from dark brown to light brown. For biobleaching with static incubation, pulp decolorization was observed on day 3. In contrast, during biobleaching with shaking, pulp decolorization occurred on day 1; further, the color of the pulp returned to dark brown. Biobleaching with shaking reduced the incubation time from 3 to 1 d. The Kappa number reduction was also increased by the shaking incubation, by about 2 to 3-fold, compared with the static incubation (Table 1). Interestingly, the use of sterile or non-sterile enzyme preparations, with shaking, was not noticeably different on the kappa number reduction. Moreover, none of the tested conditions (the static or shaking incubation and the sterile or non-sterile enzyme preparation) greatly differed in the viscosity or physical properties of the A-OKP (Tables 2 through 4), with the exception of slight increase in brightness (1.5-fold increase) when a sterile enzyme preparation was used (Table 1). Compared with the control samples, the physical properties of all pulp samples were improved by the enzymatic treatment and were further improved after an alkaline peroxide extraction.

Using the combined extracellular enzymes for A-OKP biobleaching and shaking resulted in a greater in Kappa number reduction, and a slightly increased brightness, than biobleaching with a static incubation. The A-OKP decolorization was more rapid during shaking (1 d) than during a static incubation (3 d). It is not entirely clear how the shaking affects the biobleaching efficacy. This effect may be due to the ease of diffusion and penetration of the enzymes into the pulp, and increased oxygen transfer in the culture medium. Swamy and Ramsay (1999) reported that shaking shortened the incubation time during decolorization of a textile dye, amaranth, from 20 d to 1 d. It is important to monitor the pulp color change during biobleaching because the pulp can turn back to dark brown, leading to an increase in Kappa number and decrease in brightness (data not shown). The decolorization of pulp fibers is further supported by the observation in the differential interference microscopy (Fig. 4). Compared to untreated pulp, pulp after enzymatic treatment showed lighter in fibers color.

In the current study, a biobleaching process was developed to improve the Kappa number reduction in A-OKP, by incorporating an alkaline peroxide extraction step after the enzymatic treatment. The maximum extent of Kappa number reduction obtained with a combination of the extracellular enzymes of *I. lacteus* and *L. tigrinus*, and a subsequent alkaline peroxide extraction was approximately 26%. The results of this study confirm that the Kappa number reduction is increased when A-OKP is treated with enzymes, followed by a subsequent alkaline peroxide extraction, over what was observed when A-OKP was treated with enzymes and washed with distilled water (Afrida *et al*. 2014). It is likely that the alkaline peroxide extraction activates the removal of residual lignin from A-OKP. An enzymatic treatment with subsequent alkaline peroxide extraction or peroxide extraction is an effective method for greatly improving the Kappa number reduction (Ehara *et al*. 1997; Chakar and Ragauskas 1999) and pulp properties (Babot *et al*. 2011).

The cellulase activity of the pooled extracellular enzyme fractions of *I. lacteus* and *L. tigrinus* was 99 ± 1.8 $\mu mol \cdot L^{-1} \cdot min^{-1}$. The high cellulase activity in the combined

extracellular *I. lacteus* and *L. tigrinus* enzyme preparation adversely affected the enzyme-treated pulp, as demonstrated by the fact that pulp viscosity and physical properties were not significantly reduced after the enzymatic treatment.

Viscosity is an indirect indication of the cellulose quality (Moreira *et al*. 2003). Some reduction of pulp viscosity is associated with the most oxidative pulp delignification and bleaching treatment. A pronounced reduction in pulp viscosity negatively affects the pulp quality; however, the decrease of pulp viscosity during treatment with extracellular enzymes *I. lacteus* and *L. tigrinus* were moderate (33 to 119 mLg^{-1}). Similar results were reported by Ibarra *et al.* (2006), who treated the pulp with a Lac mediator, with a 100 mLg^{-1} loss of viscosity.

Table 1. Brightness, Viscosity, and Kappa Number of the Pulp after an Enzymatic Treatment and a Subsequent Peroxide Extraction

Parameter	Control	Statically (3 d) (non-sterile)	Shaking (1 d) (non-sterile)	Shaking (1 d) (sterile)
Brightness (% ISO)	69.1 ± 0.2	69.3 ± 0	69.1 ± 0.3	70.7 ± 0.2
Viscosity ($mL \cdot g^{-1}$)	1194 ± 73	1075 ± 8	1118 ± 5	1161 ± 19
Kappa number reduction (%)	-	23.1	25	26

Table 2. Physical Properties of the Pulp after an Enzymatic Treatment with Shaking under Non-Sterile Conditions and a Subsequent Peroxide Extraction

Property	Control	Shaking (non-sterile)	Gain (%)
Tensile index ($N \cdot m \cdot g^{-1}$)	14.8 ± 0.5	17.8 ± 0	20
Burst index ($kPa \cdot m^2 \cdot g^{-1}$)	4.0 ± 0.2	5.4 ± 0.3	35
Tear index ($mN \cdot m^2 \cdot g^{-1}$)	4.2 ± 0.0	4.7 ± 0.1	12

Table 3. Physical Properties of the Pulp after an Enzymatic Treatment with Shaking under Sterile Conditions and a Subsequent Peroxide Extraction

Property	Control	Shaking (sterile)	Gain (%)
Tensile index ($N \cdot m \cdot g^{-1}$)	14.8 ± 0.5	17.7 ± 0.2	20
Burst index ($kPa \cdot m^2 \cdot g^{-1}$)	4.0 ± 0.2	5.0 ± 0.1	25
Tear index ($mN \cdot m^2 \cdot g^{-1}$)	4.2 ± 0	4.0 ± 0.1	-5

Table 4. Physical Properties of the Pulp after an Enzymatic Treatment with Static Incubation under Non-Sterile Conditions and a Subsequent Peroxide Extraction

Property	Control	Static (non sterile)	Gain (%)
Tensile index ($N \cdot m \cdot g^{-1}$)	14.8 ± 0.5	19.5 ± 0.5	32
Burst index ($kPa \cdot m^2 \cdot g^{-1}$)	4.0 ± 0.2	5.9 ± 0.3	48
Tear index ($mN \cdot m^2 \cdot g^{-1}$)	4.2 ± 0	5.5 ± 0.2	31

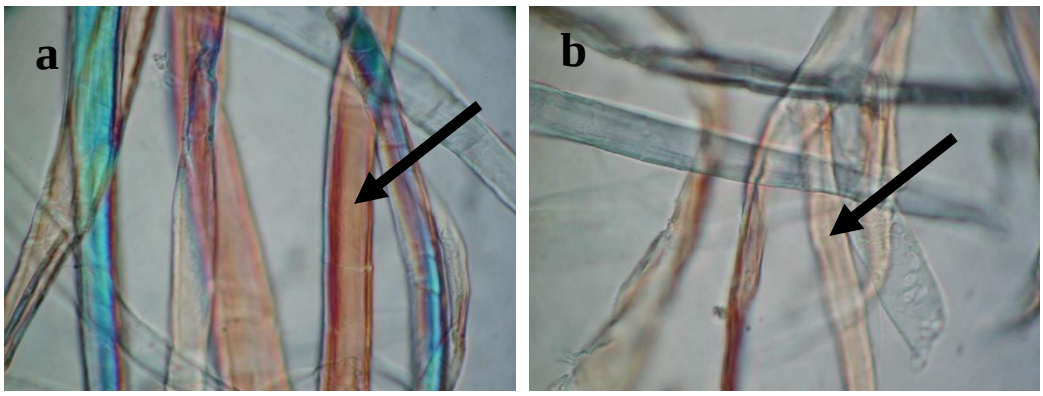

Fig. 4. Decolorization of A-OKP pulp fibers was observed by using differential interference microscopy. Untreated pulp (a) and treated pulp with extracellular enzymes (b). Black arrow indicates the fiber of the pulp.

The utilization of agricultural wastes for lignolytic enzyme production has attracted increasing attention (Elisashvili *et al.* 2006). Lignolytic enzymes have various potential applications in the chemical, fuel, food, pulp and paper, textile, and animal feed industries. Low-cost production is imperative for the employment of lignolytic enzymes on an industrial scale (Cabaleiro *et al.* 2002). One solution is to use agricultural and forestry waste for extracellular lignolytic enzyme production, for example, during submerged solid-state fermentation (Rivela *et al*. 2000; Elisashvili *et al.* 2006). However, the nutrition plays an important role in extracellular enzyme production, as the type and composition of the lignocellulosic substrate determine the type and amount of enzyme produced (Mikiashvili *et al.* 2004; Moldes *et al.* 2004).

A previous study (Afrida *et al*. 2014) demonstrated that wood rice bran glucose (WRBG) medium has the potential to be used as a culture medium for the production of stable extracellular enzymes without increasing the kappa number. We suggest that this stability is due to the secretion of different natural co-factors such as organic acids in culture medium. Organic acids (malonate and oxalate) produced by *I. lacteus* and *L. tigrinus* in WRBG culture medium are higher than in wood rice bran (WRB) and wood rice bran malonate (WRBM). According to Wariishi *et al.* (1992), organic acid plays an important role in MnP reactions because they dissociate of Mn^{3+} bound from the enzyme complex and stabilize Mn^{3+} in aqueous solutions. In the same study, they find that from several chelator agents tested, malonate show the most effective chelator for MnP activity. A study of biobleaching with semipurified MnP has shown that Mn^{3+}-malonate complexes are responsible to the brightening and delignification of kraft pulp (Kondo *et al*. 1994). Thus, the high concentrations of malonate and oxalate secreted by *I. lacteus* and *L. tigrinus* in WRBG culture may correlate with activities and stabilities of delignifying extracellular enzymes.

However, the use of WRBG medium should be re-evaluated on account of the increasing cost of glucose. One possible substitute of glucose, palm sugar, which may be easily obtained from traditional markets in Indonesia, can be considered as carbon source for microbial extracellular lignolytic production. The calculated cost of 1 L of WRBG substrate is US $0.21; the calculated cost of 1 L of WRBP substrate is US $0.03. The potential application of WRBP as a culture medium for the large-scale production of extracellular lignolytic enzymes might therefore offer a remarkable economical advantage.

The present study demonstrated the potential of employing extracellular enzymes produced by *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 in the biobleaching of A-OKP.

CONCLUSIONS

1. This study demonstrated the potential of employing extracellular enzymes produced by *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 in the biobleaching of A-OKP. The use of extracellular enzymes, with a subsequent alkaline peroxide extraction, resulted in a further improvement kappa number reduction, compared with a previous study (Afrida *et al.* 2014).
2. The performance of biobleaching with extracellular enzymes is improved with shaking and the latter reduces the incubation time by 48 h.
3. The use of sterile vs. non-sterile enzyme preparations did not noticeably affect the kappa number reduction, viscosity, and pulp properties of A-OKP.
4. The low cost of the WRBP medium may offer an economic advantage for large-scale enzyme production.

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