Comparison of the Ability of Several White-Rot Fungi to Biobleach *Acacia* Oxygen-delignified Kraft Pulp

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ABSTRACT

Previous screening analyses demonstrated that the *in vivo* biobleaching activities of the white-rot fungi *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 are higher than those of *Phanerochaete chrysosporium* and *Trametes* versicolor. The purpose of the current study was to examine the production of extracellular enzymes of these four white-rot fungi grown on three types of low-cost media containing agricultural and forestry waste, and to evaluate the ability of the produced extracellular enzymes to biobleach *Acacia* oxygen-delignified kraft pulp (A-OKP). The biobleaching activity of extracellular fractions of *I. lacteus*, *L. tigrinus*, *T. versicolor*, and *P. chrysosporium* cultures was the most pronounced after 3 days of incubation with *Acacia mangium* wood powder supplemented with rice bran and 1% glucose (WRBG) with resultant Kappa number reduction of 4.4%, 6.7%, 3.3%, and 3.3%, respectively. Therefore, biobleaching ability of *I. lacteus* and *L. tigrinus* have been shown to be higher than of *T. versicolor* and *P. chrysosporium*, both *in vivo* and *in vitro*.

Keywords: white-rot fungi; Acacia kraft pulp; Biobleaching; Kappa number

1. INTRODUCTION

White-rot fungi are natural decomposers. They generally dwell on dead hardwood rather than softwood, and decompose the main components of wood, including cellulose, hemicelluloses, and lignin, to carbon dioxide and water [1]. However, a few species are unique because they selectively remove lignin from wood without extensive cellulose degradation [2], with the wood appearing white and fibrous [3]. When these fungi decompose lignocelluloses, they typically produce a series of enzymes (hydrolases, oxidoreductase, lignolytic enzymes), and rely on nonenzymatic mechanisms [4]. From the various enzymes secreted by white-rot fungi, lignolytic enzymes are usually studied with respect to their biotechnological application because of their ability to degrade lignin and aromatic compounds [5,6]. Hence, the use of lignolytic enzymes in biotechnology has

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attracted considerable attention, and stimulated screening and selection of promising white-rot fungi as enzymes producers. Moreover, obtaining large amounts of low-cost lignolytic enzymes requires the identification of the suitable substrates.

Among the white-rot fungi, *Phanerochaete chrysosporium* and *Trametes versicolor* are the most extensively studied lignin-degrading basidiomycetes. This is because they produce large amounts of extracellular lignolytic enzymes. The utility of both fungi for various industrial applications has been widely studied, e.g., environmental pollution [7,8], in pulp and paper industry [9,10], and for animal feed production [11]. Therefore, two fungi are commonly used as species standards when comparing the potential of new strains to produce lignin-degrading enzymes [12,13]. Previously, the ability of 600 white-rot Indonesian fungal sources to biobleach *Acacia* oxygen-delignified kraft pulp (A-OKP) was screened, with *P. chrysosporium* and *T. versicolor* as standard fungi [14]. The biobleaching *in vivo* activity of the selected fungal strains *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 was higher than that of *P. chrysosporium* and *T. versicolor*. The bleaching stage of pulp processing is one of the processes that contribute the most to environment pollution in the pulp and paper industry. The commonly used chlorine-based chemical agents can react with residual lignin to produce organochlorine compounds, which are toxic, mutagenic, persistent, and bioaccumulating, and which harm biological systems [15]. Enzymes from white-rot fungi have potential applications to reduce dangerous chemical agents in the pulp and paper industry.

The aim of the current study was to continue from the previous study, to evaluate the A-OKP biobleaching activities of extracellular enzymes produced by white-rot fungi (*T. versicolor*, *P. chrysosporium, I. lacteus* KB-1.1. and *L. tigrinus* LP-7) using low-cost media containing agricultural and forestry waste.

2. MATERIAL AND METHODS

2.1. Fungal strains

T. versicolor (NBRC 30340) and *P. chrysosporium* (NBRC 31249) were provided by the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC, Japan). *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 were isolated and identified as described elsewhere [14]. All fungi were maintained at 30°C on agar slants containing 0.2% *Acacia mangium* wood powder, 0.01% guaiacol, and 1.6% potato dextrose agar (PDA).

2.2. Extracellular enzyme production

Time-course of lignolytic enzyme production of the white-rot fungi was performed on shallow static liquid cultures in 100 mL Erlenmeyer flask at 30 °C. The pre-inoculum was prepared by growing the fungi on PDA at 30 °C for 7 days. The single plugs of pre-inoculum (6 mm diameter) from the 1-week-old colonized plate were inoculated with 10 mL of media. Three type media was used for the experiments: the basal medium (2% of *A. mangium* wood powder and rice bran (3:2) in distilled water, WRB), *A. mangium* wood powder and rice bran in 1% of aqueous solution of glucose (WRBG), and *A. mangium* wood powder and rice bran in malonate buffer containing 50 mM $MnSO_4 \cdot 5H_2O$ (WRBM) [16]. Every 3 days for 21 days, the cultures were harvested, the solids were separated by filtration followed by centrifugation at 10,000 rpm (r = 55 mm), at 4°C for 10 min. The supernatants were used for enzyme activity assays.

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2.3. Enzyme assays

Lignolytic enzyme activities of Manganese peroxidase (MnP), Manganese-independent peroxidase (MIP), and Laccase (Lac) were determined by measuring the absorbance change at 470 nm related to the rate of oxidation of 2,6-dimethoxyphenol in malonate buffer (pH 4.5) [16]. Meanwhile, Lignin peroxidase (LiP) activity was determined by the oxidation of veratryl alcohol in succinate buffer (pH 3.0) at 310 nm.

The xylanase activity was assayed using a modified method of Bailey *et al.* [17] with oat spelt xylan (1%) as a substrate at 50 °C and pH 5.3. Cellulase activity was determined by reducing sugars from carboxymethy cellulose (2% w/v, low viscosity). Xylose and glucose standard curves were used to calculate the xylanase and cellulase activities [18].

2.4. Biobleaching treatment

The biobleaching activity of extracellular enzymes secreted by the four white-rot fungi on WRB, WRBG, and WRBM was evaluated on A-OKP, which had an International Standard (ISO) brightness of 47.6% and Kappa number of 9. The experimental treatments were performed in 25-ml screw cap bottles, stationary reactions, and incubated at 40°C for 1, 2, 3, and 4 days [16].

2.5. Pulp characterization

Pulps treated enzymes were determined in term their Kappa number according to micro kappa number [19].

3. RESULTS

3.1. Extracellular lignolytic enzyme activities

The time-courses of the production of lignolytic enzymes by *T. versicolor* and *P. chrysosporium* are presented in Fig. 1. *T.* versicolor produced MnP, MIP, and Lac, but not LiP. The MnP activity was the dominant lignolytic enzyme activity produced by this fungus in the WRB, WRBG, and WRBM culture media; it was highest in the WRBM medium. The second highest lignolytic enzyme activity was MIP, and the lowest lignolytic activity was Lac. The lignolytic activities of MnP, MIP, and Lac were 2- to 3-fold higher in the WRBM medium than in the WRB medium (the basal medium). On the other hand, *P. chrysosporium* produced MnP, MIP, Lac, and LiP on WRB and WRBG; no MnP, MIP, and Lac activities were detected on WRBM. LiP was the highest detected lignolytic activity of *P. chrysosporium*.

In the current study, the pH of the culture medium during the mycelial growth of *T*. *versicolor* and *P*. *chrysosporium* was determined (Fig. 2). The pH of *T*. *versicolor* and *P*. *chrysosporium* WRB cultures was *ca.* 5–6. In WRBG, the pH was initially 6 and gradually decreased to 4, except for *P. chrysosporium* cultures, where it was initially 6, then gradually decreased to 4, and increased to 6 at the end of incubation. In WRBM, the pH was initially 4.5 and increased gradually over the course of incubation to 6–7.

3.2. Biobleaching treatment

In the current study, Kappa number reduction was used as an indicator of delignification. The biobleaching activity and stability of the four white-rot fungi on WRB, WRBG, and WRBM are shown in Fig. 4. On the WRB medium, a slight increase in Kappa number reduction of the pulp was observed on day 1 with *P. chrysosporium*, while Kappa number reduction from the three other fungi was small. The highest Kappa number reduction by *P.*

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chrysosporium and *L. tigrinus* enzymes occurred on day 2, and on day 3 for *T. versicolor* and *I. lacteus* enzymes. However, all fungi remained stable (without increasing in Kappa number values) on day 4. The Kappa number reduction upon incubation with the WRBG culture supernatant was stable, except for *P. chrysosporium* culture supernatant, in which it smaller on days 2 and 4. Remarkably, for reactions with enzymes from all fungi grown in the WRBG culture medium, the Kappa number reduction increased on day 3. In reactions containing the WRBM culture medium, the Kappa number reduction stably increased on day 1, but the Kappa number reduction varied on other days. From the three types of media, incubation with the WRBG culture supernatant resulted in a greater than expected Kappa number reduction, as compared with the WRB and WRBM culture media.

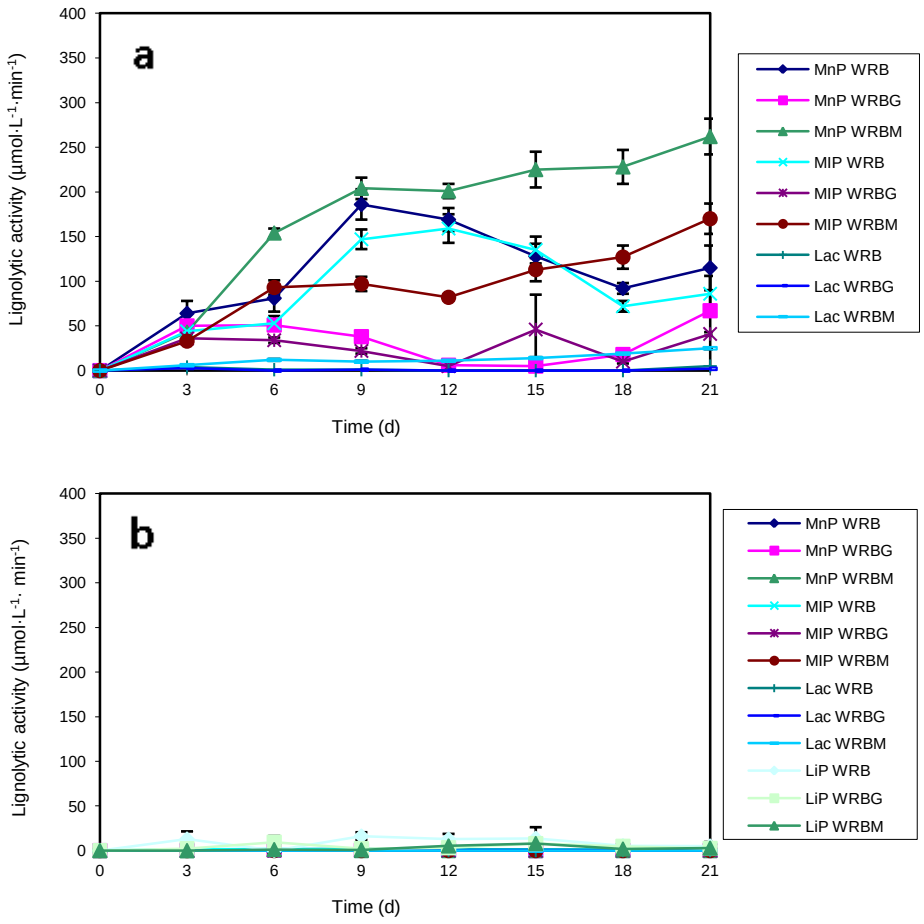

Fig. 1. Time-courses of lignolytic enzyme production by *T. versicolor* (a) and *P. chrysosporium* (b) grown in shallow stationary liquid culture on WRB, WRBG, and WRBM.

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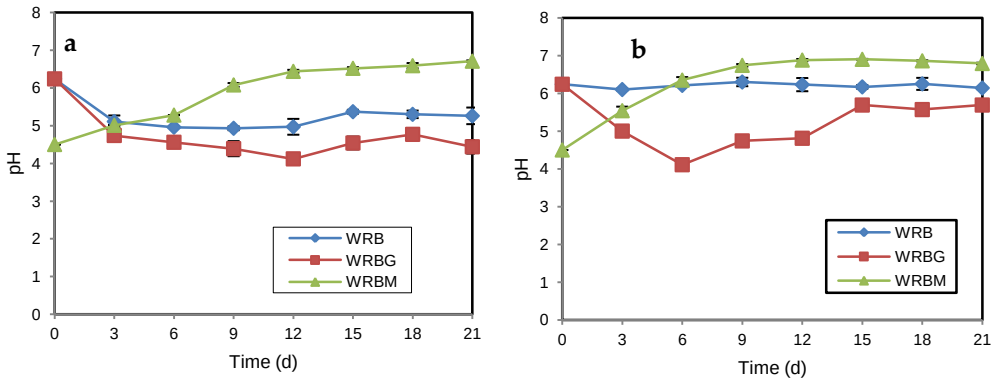
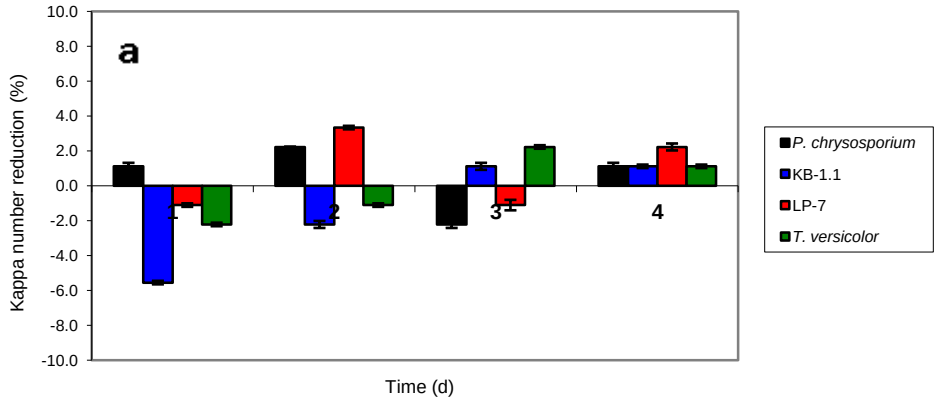
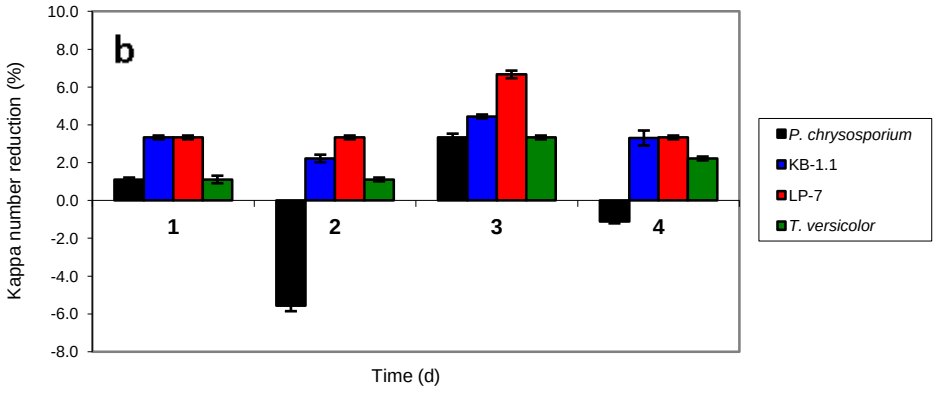


Fig. 2. Changes in the *T. versicolor* (a) and *P. chrysosporium* (b) culture pH during lignolytic enzyme production.





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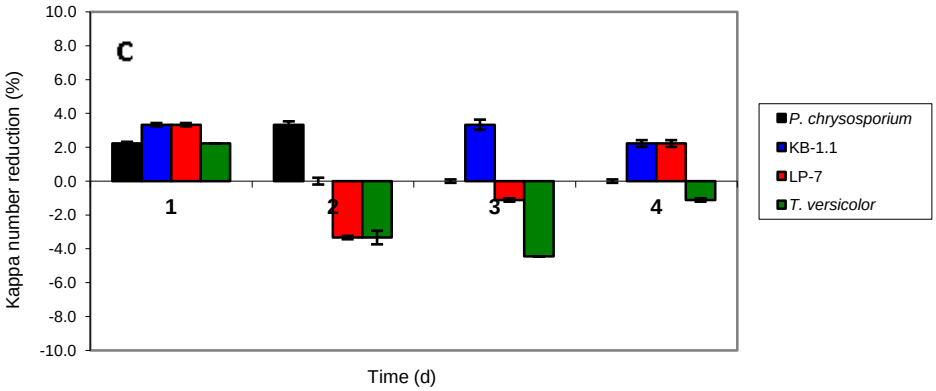
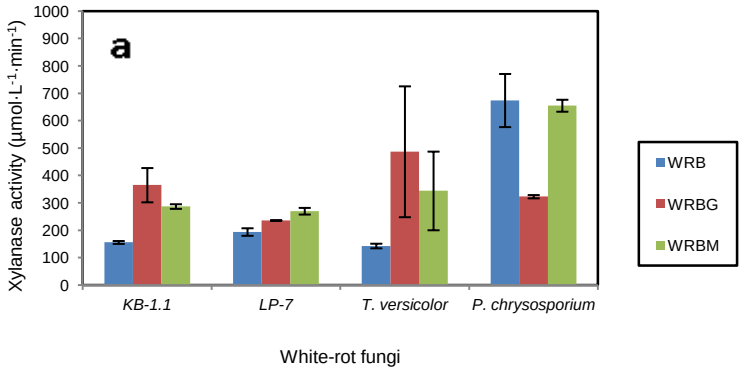


Fig. 3. Biobleaching activities of the white-rot fungi grown on three different media: (a) WRB, (b) WRBG, and (c) WRBM.

The xylanase and cellulase activities in culture media that exhibited maximal MnP activity were then determined. The xylanase is responsible enzyme for xylan depolimerization. Xylanase present in the culture medium may participate in biobleaching. As shown in Fig. 4a, the highest xylanase activity in the WRB and WRBM media was detected in the *P. chrysosporium* cultures, while the highest xylanase in the WRBG medium was detected in the *T. versicolor* culture. *L. tigrinus* produced less xylanase activity than other white-rot fungi. Further, cellulase activity of the white-rot fungi was triggered by glucose and inhibited by malonate (Fig. 4b). The highest cellulase activity was associated with *T. versicolor*.



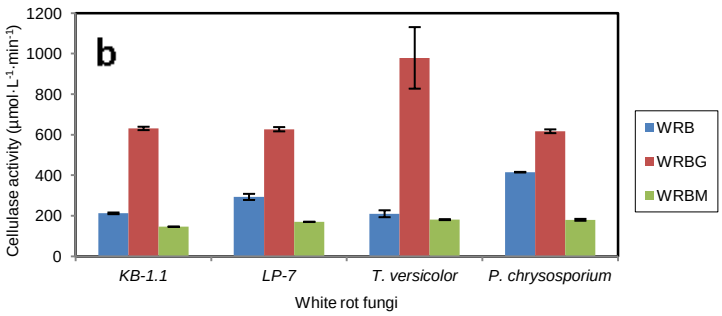


Fig. 4. Xylanase activity (a) and Cellulase activity (b) in extracellular enzyme fractions with maximum MnP activity that were used for A-OKP bioleaching.

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4. DISCUSSION

The biobleaching activity of white-rot fungi is correlated with their extracellular lignolytic enzymes, together with low molecular-weight cofactors [20]. To date, four families of lignolytic enzymes have been shown to be involved in biobleaching: LiP, MnP, Lac, and MIP. To determine their extracellular enzyme activities, the selected white-rot fungi, were grown in shallow static liquid cultures and the enzyme activity was measured.

The time-courses of the production lignolytic enzymes of *T.* versicolor and *P. chrysosporium* have been shown in Fig. 1. Meanwhile, time-courses for *I. lacteus* and *L. tigrinus* refer to report previously [16]. We compared the production of lignolytic enzymes among the white-rot fungi, the results showed that *T.* versicolor, *I. lacteus* and *L. tigrinus* produced MnP, MIP, and Lac, but no LiP. Meanwhile, *P. chrysosporium* produced highest LiP activities in all media. It has been reported that lignolytic enzyme production depends on the fungal species, the lignocellulosic growth substrate, and the cultivation method [21]. *T. versicolor* is a well-known fungal species that produces MnP and Lac [4]; however, the secretion of enzymes depends on the *T. versicolor* strain [22,23]. Hossain and Anantharaman reported that *T. versicolor* produces MnP, LiP, and Lac on bagasse powder in liquid shake cultures [24]. On the other hand, *P. chrysosporium* is not attractive for industrial-scale use because it requires complex physiological conditions for lignin degradation [25]. However, until recently, researchers have attempted to improve the lignolytic enzyme production in *P. chrysosporium* [26,27]. *I. lacteus* produces MnP, Lac, and LiP in a non-immersed liquid culture under nitrogen limitation condition [28], whereas *L. tigrinus* produces MnP and Lac in the substrate during growing, but no LiP secretion [29,30].

The A-OKP ability of the extracellular enzymes produced on three types of culture media (WRB, WRBG, and WRBM) was assessed. The extracellular enzymes were collected from cultures that exhibited maximum MnP activity, except for *P. chrysosporium*, which was, collected during maximum LiP activity. According to previous studies, MnP underpins the biobleaching ability of various white-rot fungi [31-33]. Thus, MnP is more important for delignification during biobleaching than other lignolytic enzymes [34,35]. For *T. versicolor*, the maximum MnP activity in WRB was 169 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 12 days); in WRBG it was 67 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 21 days); and in WRBM it was 262 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 21 days). Meanwhile, the maximum MnP activity of *I. lacteus* and *T. tigrinus* was as reported previously [16]. On the other hand, LiP was the highest detected lignolytic activity of *P. chrysosporium*: in WRB it was 16.2 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 9 days); in WRBG it was 9.4 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 6 days); and in WRBM it was 7.9 $\mu mol \cdot L^{-1} \cdot min^{-1}$ (after 15 days). From the three types of media, incubation with the WRBG culture supernatant resulted in a greater than expected Kappa number reduction, as compared with the WRB and WRBM culture media. The previous study has been shown that *I. lacteus* and *T. tigrinus* produces high organic acids (malonate and oxalate) in WBRG [16]. We suggest that the presence of organics acids in culture media containing glucose could be correlated with performance of lignolytic enzymes of white-rot fungi to degrade lignin residues in A-OKP. Organic acids have known important in lignin degradation reactions including as an effective chelator for MnP activity [35]. Decreasing pH of *T. versicolor* from initial pH 6 to 4 may (Fig. 2) correlate with the production of organic acids.

The increasing Kappa number values phenomenon may reflect possible lignin modification and/or repolymerization by the enzymes. This case was also reported previously for Lac-mediated systems that were used to bleach eucalyptus kraft pulp [36]. Such increase suggested that Lac is involved in the polymerization and grafting of phenolic compounds in the presence of syringaldehyde, promazine, and vanillin as mediators. However, Kappa number reduction of A-OKP was improved after treated with enzymes, followed by alkaline

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peroxide extraction [37], compared to A-OKP treated with enzymes and washed with distilled water [16].

The use of xylanase as a bleaching agent has been reported in many studies, which demonstrated that xylanase use can reduce the need for chemical agents at the bleaching stage [38]. According to Elisashvili, some white-rot fungi are excellent producers of xylanase and cellulase under appropriate cultivation conditions [21]. Accordingly, *P. chrysosporium* and *T. versicolor* were good xylanase producers on the base wood rice bran medium. High cellulase activity in pooled extracellular enzymes used for biobleaching should be avoided because it may degrade the cellulose to pulp fiber, consequently negatively affecting the physical properties of the pulp. However, an appropriate amount of cellulase activity in the culture medium does not affect the viscosity and physical properties of the enzyme-treated pulp [37].

5. CONCLUSION

1. The presence of malonate in the basal medium (wood powder rice bran, WRB) enhanced the activity of lignolytic enzymes, while the presence of glucose in the basal medium suppressed the activity of these enzymes.
2. Extracellular enzyme-containing supernatant of WRB, WRBG, and WRBM cultures was used in A-OKP biobleaching. The Kappa number reduction was more stable and higher when WRBG supernatant was used than when WRB or WRBM supernatants were used.
3. The stability and performance of the lignolytic enzymes, and their cofactors, in the culture medium affected the performance of extracellular enzymes in A-OKP biobleaching to a greater extent than a robust production of the lignolytic enzymes as such.

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COMPETING INTERESTS

No

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author SA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors TW and YT managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

CONSENT (WHERE EVER APPLICABLE)

No need

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

No need

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