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ORIGINAL PAPER

Biobleaching of *Acacia* kraft pulp with extracellular enzymes secreted by *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 using low-cost media

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Abstract The white-rot fungi *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 have been shown in previous studies to have high biobleaching activity in vivo. The aim of this study was to investigate the activities and stabilities of extracellular enzymes, prepared from *I. lacteus* and *L. tigrinus* culture grown in three types of economical media of agricultural and forestry wastes, for biobleaching of *Acacia* oxygen-delignified kraft pulp using kappa number reduction as an indicator of delignification. After 3 days of incubation, the extracellular enzymes preparations from *I. lacteus* and *L. tigrinus* cultures in media of *Acacia mangium* wood powder supplemented with rice bran and addition 1 % glucose (WRBG), resulted in significant decrease of 4.4 and 6.7 %, respectively. A slightly higher kappa number reduction (7.4 %) was achieved with the combine extracellular enzymes from *I. lacteus* and *L. tigrinus*. One of the strategies for reducing the cost of enzyme production for treatment processes in the pulp and paper industry is the utilization of agricultural and forestry waste. Thus, WRBG has potential as a culture medium for producing stable lignolytic enzymes simply and economically.

Keywords *Acacia* kraft pulp · Biobleaching · Economical medium · Extracellular enzymes

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Introduction

Enzymes from microorganisms have potential applications in the pulp and paper industry. These enzymes can reduce the use of chlorinated and other compounds utilized for chemical bleaching, as well as improving the quality of the pulp. Currently the most important application is the pre-bleaching of kraft pulp.

White-rot fungi have been recognized for their ability to completely degrade all ligninocellulosic materials to carbon dioxide and water (Boyle et al. 1992). The ability of some white-rot fungi to selectively degrade lignin has made them of increasing interest for biotechnological bleaching. The ability of white-rot fungi to degrade lignin is associated with their extracellular lignolytic enzymes, together with low-molecular-weight cofactors (Leonowicz et al. 1999). To date, four families of lignolytic enzymes are known to be involved in lignin degradation: manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), manganese-independent peroxidase (MIP) and laccase (Lac). The activities of MnP, LiP and Lac have been correlated with increased brightness of kraft pulp (Katagiri et al. 1995). MIP was discovered in the white-rot fungus *Bjerkandera* sp. BOS55 (De Jong et al. 1992) and was shown to be potentially involved in biobleaching (Moreira et al. 2001).

Many researchers consider MnP to be most important lignolytic enzyme for kappa number reduction. A partial correlation between MnP activity and increased brightness has been found during the biobleaching process (Moreira et al. 1997). In addition, MnP-deficient mutants of *Trametes versicolor* are not able to cause bleaching and bleaching activity can be restored by the exogenous addition of MnP (Addleman et al. 1995). Consequently, research on MnP purification for biobleaching applications has intensified. Kondo et al. (1994) have reported that

purified MnP can increase brightness in the presence of $MnSO_4$, Tween 80, and sodium malonate with the continuous addition of H_2O_2. Harazono et al. (1996) purified MnP from the fungus *Phanerochaete sordida* YK-624 and were able to increase brightness in the presence of an organic acid such as oxalate. Thus, although MnP is a good enzyme for degrading residual lignin in pulp, it requires additional elements. A previous study has shown that the degradation of residual lignin in pulp is not due to MnP alone, but also to the present of Lac during the bleaching process (Reid and Paice 1994a). In addition, Westermark and Eriksson (1974) have reported that the degradation of lignin by white-rot fungi in nature is an interdependence system. Thus, all of the fungal enzymes cooperate to degrade wood components in nature. This must be considered when purifying specific lignolytic enzymes for biobleaching applications.

Reducing the cost of enzyme production is required for developing enzymatic treatment processes in the pulp and paper industry. The use of crude enzymes in biobleaching of loblolly pine kraft pulp has shown promising results (Re et al. 2008). This is because white-rot fungi produce hemicellulases (xylanase or mannanase) in addition to lignolytic enzymes and the medium also may include other proteins or factors that might stabilize the extracellular enzymes. However, nutrition also plays an important role in extracellular enzyme production. Commercial products such as malt extract, peptone, some vitamins, and minerals, are used in conventional fungal media and can be expensive if the enzymes are to be produced on a large scale. Residual agricultural and forestry wastes, such as rice bran and wood powder, could be used as sources of carbon, vitamins and minerals for producing extracellular enzymes and are accessible, easy-to-handle, and economical.

The present study evaluates the activities and stabilities of extracellular enzymes produced by *Irpex lacteus* KB-1.1 and *Lentinus tigrinus* LP-7 for biobleaching of *Acacia* oxygen-delignified kraft pulp (A-OKP). Our previous study with an in vivo system has shown that the biobleaching activities of *I. lacteus* and *L. tigrinus* greatly exceed those of the highly lignin-degradative fungi *Phanerochaete chrysosporium* and *T. versicolor* (Afrida et al. 2009). To our knowledge, this was the first use of *I. lacteus* for biobleaching and there have been only rare reports of biobleaching with *L. tigrinus* (Moreira et al. 1997).

Materials and methods

Fungal cultures

Irpex lacteus KB-1.1 and *L. tigrinus* LP-7 were maintained on agar slants at 30 °C. The medium was prepared with 0.2 % *Acacia mangium* wood powder, 0.01 % guaiacol and 1.6 % agar (Nishida et al. 1988). The pre-inoculum was obtained by incubating the fungi on potato dextrose agar (PDA) at 30 °C for 7 days.

Culture media and enzyme production

Extracellular enzyme production was carried out at 30 °C in shallow stationary liquid cultures (10 ml media in 100-ml Erlenmeyer flasks). The basal medium was 2 % *A. mangium* wood powder (40–60 mesh) and rice bran (3:2) in distilled water (WRB). The effect of replacing the distilled water with a 1 % aqueous solution of glucose (WRBG) was evaluated. To study the effect of manganese (Mn^{2+}) in malonate buffer, 330 μM $MnSO_4.5H_2O$ in 50 mM malonate buffer, pH 4.5, (WRBM) was substituted for distilled water. The flasks were inoculated with single plugs (6 mm diameter) from 1-week-old cultures grown on PDA and incubated without shaking at 30 °C. Every 3 days for 21 days, cultures were filtered and centrifuged at 10,000 rpm at 4 °C for 10 min and the supernatants were used for enzyme activity assays. The experiment was carried out in triplicate.

Enzyme activity assays

Enzyme activities were determined using a UV-1600 Shimadzu spectrophotometer. All the lignolytic enzymes, including MnP, Lac, LiP and MIP were assayed at 40 °C. The method of Hirai et al. (1994) was slightly modified to determine lignolytic activity. MnP, MIP and Lac activities were monitored by the oxidation of 2,6-dimethoxyphenol at A_{470}. Reaction mixtures for MnP activity contained 1 mM 2,6-dimethoxyphenol, 0.2 mM H_2O_2 and 1 mM MnSO4, in 50 mM malonate buffer (pH 4.5). Reaction mixtures for MIP activity contained 1 mM 2,6-dimethoxyphenol, 0.2 mM H_2O_2 and 1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM malonate buffer (pH 4.5). 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).

Analysis of organic acids

The extracellular fluid was collected from cultures with maximum MnP activity. Organic acids were analyzed by Capillary Electrophoresis System (Quanta 4000CE, Waters, Milford, MA, USA). The extracellular fluid was filtered with a membrane filter (pore size = 0.45 μm) and diluted 4 times with milliQ water. The standards, including

citric acid, malic acid, tartaric acid, oxalic acid, succinic acid, malonic acid, and glycolic acid, were prepared with milliQ water at concentrations of 1 and 10 mM.

Pulp treatment

A-OKP was provided by an industrial kraft pulp mill in Indonesia and had an International Standard Organization (ISO) brightness of 47.6 % and a kappa number of 9. The A-OKP was used to determine the biobleaching activity of extracellular enzymes secreted by *I. lacteus* and *L. tigrinus* on WRB, WRBG and WRBM. The extracellular enzymes were collected from cultures with maximum MnP activity, filtered and centrifuged at 10,000 rpm for 10 min at 4 °C. The A-OKP was sterilized (121 °C, 20 min) in 25-ml screw cap bottles and samples (equivalent to 1 g oven-dried weight) were added to 10 ml of extracellular enzymes. The stationary reactions were incubated at 40 °C for 1, 2, 3 and 4 days. Controls without extracellular enzymes were carried out in parallel. After treatment, the pulp samples were filtered and washed with distilled water. The pulp lignin oxidation was measured by the difference in kappa number reduction between the assays with extracellular enzymes and the parallel controls. All treatments were performed in triplicate.

To determine the optimal incubation temperature, A-OKP prepared as above, was treated with extracellular enzymes of *I. lacteus* and *L. tigrinus*, obtained from WBRG cultures with maximum MnP activity at 30, 40 and 50 °C.

The effect of the pulp pH on biobleaching was assessed at pH 9, 7 and 4.5. The initial pH of A-OKP was 9.0. This was reduced to pH 7 by washing extensively with distilled water until the filtrate was colorless. The washed pulp was submerged overnight into 50 mM malonate buffer, pH 4.5. The pulps of various pHs were treated at 40 °C for 1, 2 and 3 days with the extracellular enzymes from WRBG cultures with maximum MnP activity.

Analytical techniques

Pulp brightness was determined by using a colorimeter (Suga Test instruments Co., Ltd, Japan). This method measures the brightness of white, near-white and natural-colored pulp and paper using the directional reflectance factor at 457 nm (T 452 om-92, TAPPI 1996). The kappa number was determined according to the micro kappa number measurement (Berzins 1966). This can be used to determine the degree of pulp delignification and is the volume (in ml) of 0.1 N potassium permanganate solutions that is consumed by 1 g of moisture-free pulp under the appropriate conditions (T 236 cm-85, TAPPI 1996).

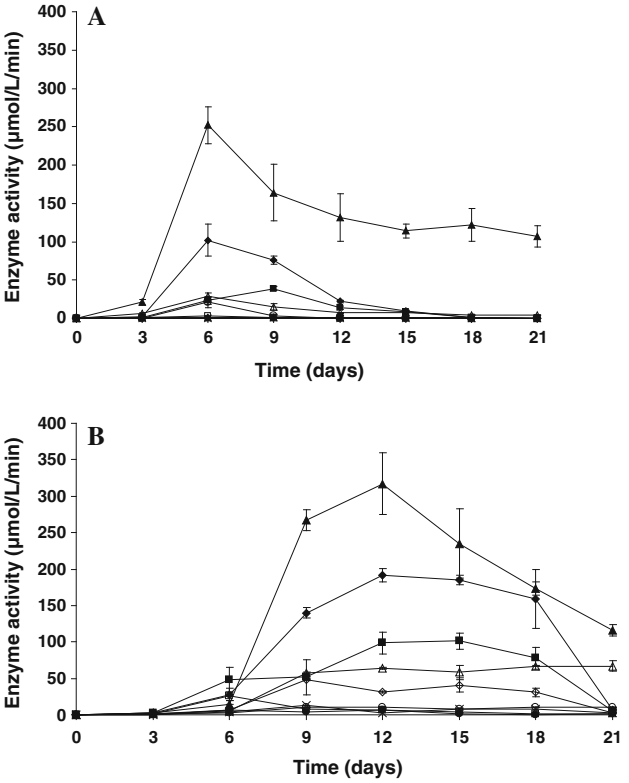

Fig. 1 Time courses of lignolytic enzyme production **a** *I. lacteus* KB-1.1 and **b** *L. tigrinus* LP-7. WRB, MnP (*filled diamond*), MIP (*open diamond*), Lac (*multiple symbol*); WRBG, MnP (*filled square*), MIP (*open square*), Lac (*filled circle*); WRBM, MnP (*filled triangle*), MIP (*open triangle*),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

Results

Extracellular lignolytic activities

The time courses of lignolytic enzymes produced by *I. lacteus* and *L. tigrinus* grown on shallow stationary liquid cultures (WRB, WRBG and WRBM) were studied. Both strains produced MnP, MIP and Lac, but not LiP, in all culture media. Compared to *L. tigrinus*, *I. lacteus* showed lower production of lignolytic enzymes. As shown in Fig. 1, *I. lacteus* and *L. tigrinus* produced MnP as the dominant lignolytic enzyme activity. The peak lignolytic activities varied greatly according to the nutritional conditions. The lignolytic activities (MnP, MIP and Lac) increased significantly by about twofold to threefold in the present of Mn^{2+} in malonate buffer (WRBM), as compared to the basal medium (WRB). *I. lacteus* and *L. tigrinus* mycelial mats also grew better on WRBM than WRB (data not shown). In contrast, the culture medium containing glucose (WRBG) suppressed the production of extracellular lignolytic enzymes of *I. lacteus* and *L. tigrinus* by twofold as compared with WRB.

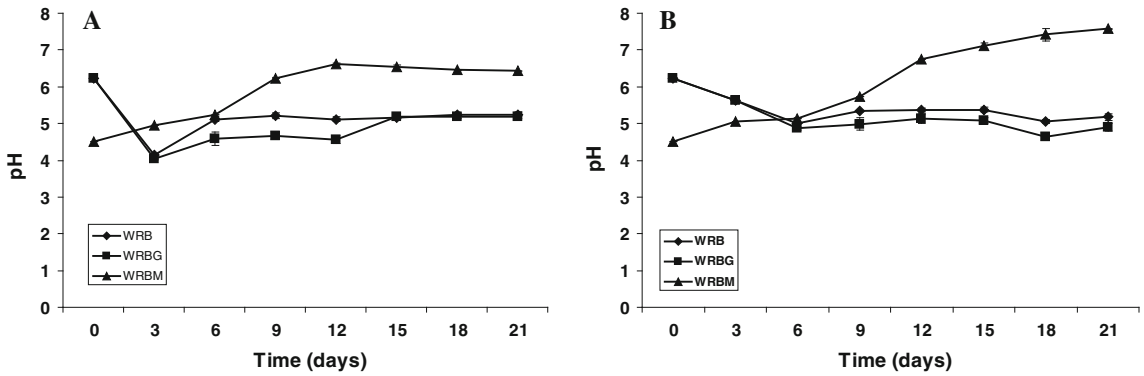

Fig. 2 Culture pH of **a** *I. lacteus* KB-1.1 and **b** *L. tigrinus* LP-7 during lignolytic enzyme production

The maximum MnP activity produced by *I. lacteus* and *L. tigrinus* varied with the medium. Maximum MnP activity of *L. tigrinus* occurred after 12 days of incubation in all media (WRBM, 317 ± 42 μmol/L/min; WRB, 192 ± 21 μmol/L/min; WRBG, 99 ± 15 μmol/L/min) whereas *I. lacteus* showed maximum MnP activity after 6 days on WRBM (252 ± 24 μmol/L/min) and WRB (102 ± 21 μmol/L/min) and after 9 days on WRBG (39 ± 3 μmol/L/min) (Fig. 1).

The rapid acidification of WRB and WRBG cultures was observed during the growth of *I. lacteus* and *L. tigrinus* with the pH reaching a value close to 5 at 21 days (Fig. 2). However, the pH of WRBM cultures increased over the course of the incubation.

Biobleaching

The maximum activity of extracellular enzymes produced by *I. lacteus* and *L. tigrinus* on WRB, WRBG and WRBM were examined for their activity and stability in the biobleaching of *Acacia* kraft pulp (Fig. 3). Kappa number reduction occurred on 3 days of incubation with enzymes from *I. lacteus* grown on WRB and remained stable on 4 days. Meanwhile, kappa number reduction occurred on 2 and 4 days of incubation with enzyme from *L. tigrinus* grown on WRB. With enzymes from *I. lacteus* grown on WRBG, the kappa number was reduced on 1st day, decreased slightly on 2nd day, rose again on 3rd day, and remained stable on 4th day. With enzymes from *L. tigrinus* grown on WRBG, a kappa number reduction was detected on 1st day, decreased slightly on 2nd day, rose sharply on 3rd day and decreased on 4th day. Delignification by enzymes from *I. lacteus* and *L. tigrinus* grown on WRBM appeared on 1st day. However, on 2nd day and 3rd day, the kappa number increased with the enzymes from *L. tigrinus*. As shown on Fig. 3, enzymes from both *I. lacteus* and *L. tigrinus* grown on WRB and WRBM resulted in increased kappa number values. These increasing kappa numbers with A-OKP were also found with the extracellular enzymes from *P. chrysosporium* and *T. versicolor* grown on WRB and WRBM but not with the enzymes from the fungi on WRBG (data not shown). Three days of incubation appeared to be the proper amount of time for observing delignification. Although the extracellular enzyme activities were lower from WRBG cultures than from WRB and WRBM cultures, the A-OKP biobleaching activity and enzyme stabilities of both of the strains were better from WRBG. These results clearly illustrate that the lignolytic enzyme activities of the cultures are not indicative of their biobleaching abilities.

Following this preliminary study, we examined the effect of temperature on biobleaching. The kappa number reduction was not improved significantly by biobleaching at 30 or 50 °C as compared with 40 °C with the combine extracellular enzymes from *I. lacteus* and *L. tigrinus* (Fig. 4A). A slight increase in kappa number reduction of the pulp was observed after 3 days of incubation at 40 °C with the combined extracellular enzymes from *I. lacteus* and *L. tigrinus* cultures grown on WRBG. These results also indicate that delignification was more stable during incubation at 40 °C than at 30 or 50 °C since the kappa number values did not increase at 40 °C.

We also determined the brightness values during the course of this experiment (Fig. 4B). Unfortunately, the reduction in kappa number was accompanied by a 2–3 point decrease in pulp brightness as compared to controls without enzymes. The initial pH of A-OKP from the mill was 9.0. The addition of the extracellular enzymes lowered the pH of the pulp to about 6.0 and this pH remained stable during four days of biobleaching (data not shown). Washing the pulp to pH 7.0 or submerging the pulp in malonate buffer to pH 4.5 did not improve the biobleaching ability or stability of the extracellular enzymes (Fig. 5).

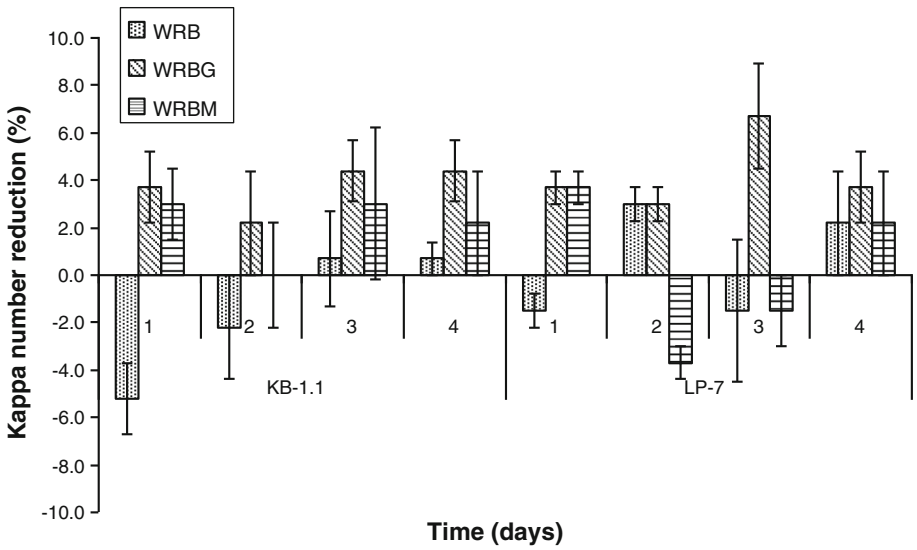

Fig. 3 The effect of different media on the kappa number reduction of A-OKP by extracellular enzymes produced by *I. lacteus* KB-1.1 and *L.tigrinus* LP-7 over four days of incubation

Organic acids

We determined the presence of organic acids in the culture media when MnP is maximal (Fig. 6). The concentration of organic acids secreted by *I. lacteus* and *L. tigrinus* were highest in culture media containing glucose. Malonate and oxalate were detected in the culture medium containing glucose, but only oxalate was found in WRB and WRBM cultures. Compared to *L. tigrinus*, *I. lacteus* showed higher production of malonate on WRBG cultures. However, *L. tigrinus* showed higher production of oxalate compared to *I. lacteus* on WRBG.

Discussion

The extracellular lignolytic enzymes of *I. lacteus* and *L. tigrinus* were examined on WRB, WRBG and WRBM media. During fungal growth, MnP was the major oxidative enzyme activity detected in the extracellular fluid with all media. As expected, manganese and organic acids such as malonate increased the secretion of lignolytic activity. Kuan and Tien (1993) reported that the oxalate content of the culture fluid correlates with MnP activity, presumably because of its ability to chelate manganese. Moreover, glycolate, malonate, glucuronate, gluconate and 2-hydroxybutyrate stimulate the production of MnP isoenzymes in the extracellular fluid (Mester and Field 1997). In addition, manganese is considered to be an inducer of MnP activity (Buswell et al. 1995; Fu et al. 1997; Papinutti and Forchiassin 2003) and the expression of the *mnp* gene in *P. chrysosporium* cultures is dependent on manganese (Brown et al. 1991). Moreover, Lac catalyzes the oxidation of manganese in the presence of the chelators oxalate and malonate (Scholosser and Höper 2002) and manganese may influence the availability of endogenous veratryl alcohol which is a known cofactor of LiP (Mester et al. 1995). Consequently, organic acids and manganese are important, directly or indirectly, for the secretion of lignolytic enzymes into the culture medium.

Rüttiman-johnson et al. (1993) reported that the activities of Lac and MnP were higher in presence of high glucose (1 %) as compared to low glucose (0.1 %). However, in our study, the addition of 1 % glucose suppressed the lignolytic activity of *I. lacteus* and *L. tigrinus* as compared with the basal medium. In general, increasing concentrations of glucose in cultures increase the fungal biomass, but inhibit the synthesis of lignolytic enzymes (Yamanaka et al. 2008).

Previous studies have shown that MnP is correlated with delignification during biobleaching (Paice et al. 1993; Katagiri et al. 1995; Addleman et al. 1995). Therefore, MnP has an important role in the delignification of residual lignin in pulp. For this reason, we used the MnP maximum for collecting the extracellular enzymes for biobleaching. The extracellular enzymes from WRBG cultures seemed to be more stable than the extracellular enzymes from WRB or WRBM cultures for biobleaching A-OKP without increasing the kappa number. We suggest that this stability is due to the secretion of different natural co-factors in WRBG medium as compared with WRB and WRBM media. These natural co-factors are important for stabilizing the oxidized enzyme product and may include such organic acids. Important roles for organic acids in biobleaching systems have been discussed by Moreira et al. (1997, 1998, 1999, 2001) and include complexing Mn^{3+}, stimulating production of extracellular lignolytic enzymes and secondary metabolites, and reducing oxygen radicals.

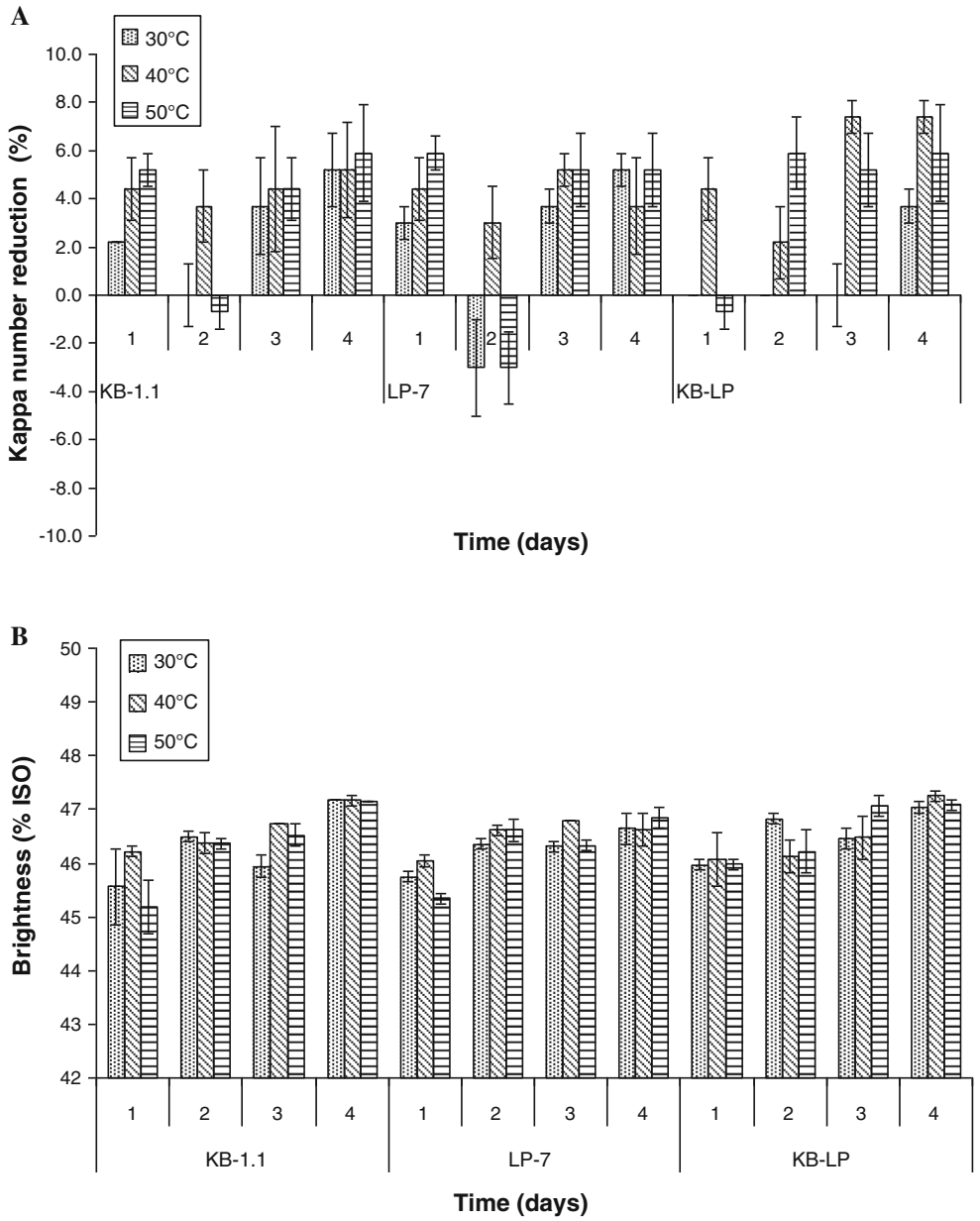

Fig. 4 Effect of temperature on kappa number reduction (**a**) and brightness (**b**) of A-OKP by extracellular enzymes from *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 cultures grown on WRBG medium and by the combined extracellular enzymes from both cultures

The high concentrations of malonate and oxalate may correlate with activities and stabilitis of delignifying extracellular enzymes from WRBG cultures (Fig. 3). The malonate buffer in WRBM was converted by *I. lacteus* and *L. tigrinus* to oxalate (Fig. 5). The increasing pH of WRBM during the growth of both fungi (Fig. 2) may have been due to the conversion of malonate to oxalate. The acidification of the WRB and WRBG culture media was observed during the growth of *I. lacteus* and *L. tigrinus.* An association between acidification of the culture medium during fungal growth and organic acid production has been reported (Mäkelä et al. 2002; Hakala et al. 2005).

Our preliminary study shows potential for the application of extracellular enzymes of *I. lacteus* and *L. tigrinus* to biobleaching of A-OKP. However, in some cases we found an increase in the kappa number and a decrease in brightness after enzymatic treatment. We suggest that this may reflect possible lignin modification and/or repolymerization by the enzymes. Further study is necessary to elucidate these phenomena. There are some reports of lignin modification and decrease brightness after treatment with lignolytic enzymes (Arbeloa et al. 1992; Reid and Paice 1994b, 1998; Wong et al. 1999). Chakar and Ragauskas (1999) have reported that the loss in brightness may

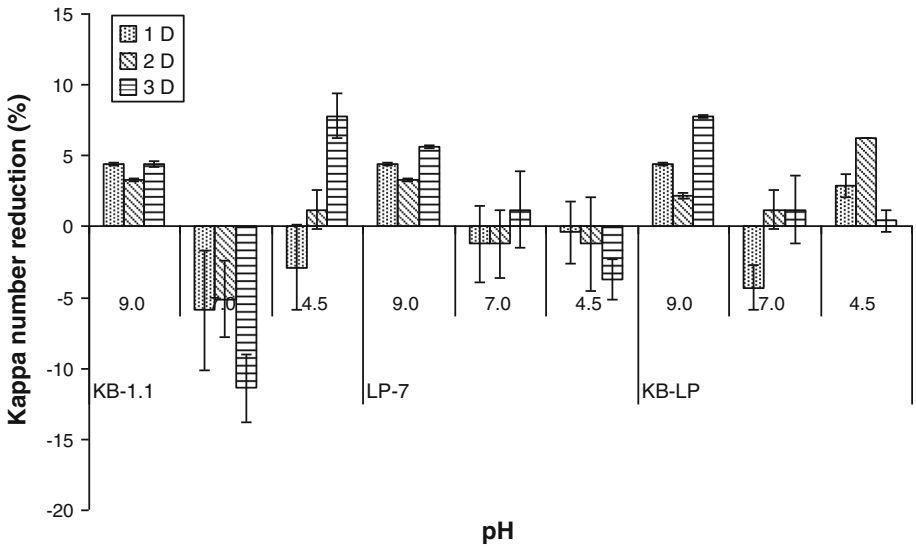

Fig. 5 pH effect on kappa number reduction of A-OKP by extracellular enzymes from *I. lacteus* KB-1.1, *L. tigrinus* LP-7 cultures grown on WRBG medium

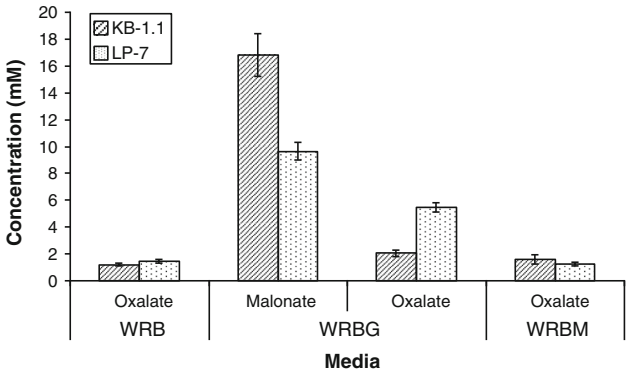

Fig. 6 Organic acids produced by *I. lacteus* KB-1.1 and *L. tigrinus* LP-7 on different media during maximal MnP activity

be attributable to quinone-type structures. Quinoids are known to be chromophores in lignin. Quinone residuals may be responsible for the darkening of pulp after enzymatic treatment and can be reversed with sodium borohydrate or repeated the enzyme treatments (Ehara et al. 1998).

The potential application of lignolytic enzymes in biotechnology will require large amounts of low-cost enzymes. One strategy is to stimulate the utilization of agricultural and forestry wastes. Wood powder and rice bran are commonly used as raw materials for culturing edible and medicinal mushrooms. Wood powder is a lignocellulosic material that is commonly used as a mushroom substrate and rice bran is an additive that can boost yield (Stamets 2000). Rice bran is known to be rich sources of carbohydrates (Hanmoungjai et al. 2000), proteins (Shih et al. 1999), lipids (Hemavathy and Prabhakar 1987), vitamins (Yasumoto et al. 1997; Diack and Saska 1994), and trace elements (Idouraine et al. 1996; Bergman et al. 1997). Cultures based on wood powder and rice bran can be used for the large-scale production of extracellular enzymes and are inexpensive, readily available and very simple. In contrast, Borràs et al. (2008) have evaluated the biotechnological cost of using *T. versicolor* and found that culturing with malt extract is too expensive to be considered. Production of lignolytic enzymes on agricultural and forestry wastes have been studied primarily by solid-state fermentation (Silva et al. 2005; Kachlishvili et al. 2006; Papinutti and Foarchiassin 2007).

Reducing the cost of enzyme production is necessary for developing enzymatic treatment processes in the pulp and paper industry. Therefore average cost of producing the extracellular enzymes in this study was evaluated. In addition to the cost of raw materials, simplicity, ease-of-handling and stability of the enzymes should be taken into consideration. We found that a medium such as WRBG, which is appropriate for extracellular enzyme production, can be obtained from agricultural waste including wood sawdust, rice bran and glucose. Cost of raw materials was used in this study including wood sawdust (US $<$ \$0.50/Kg), rice bran (US \$1.00/Kg), and glucose (US \$20.00/Kg). The calculated cost for 1 L of WRBG medium is US \$0.21. Therefore, for biobleaching, if one ton pulp without water is treated with WRBG, the cost is US \$2,100. However, if cost of WRBG is compared with malt extract (ME) known as general medium for culturing fungi, cost for 1 L ME is US \$5.37 and US \$53,700 to treat one ton pulp without water. Therefore, the use of WRBG for producing extracellular lignolytic enzymes may offer an economic advantage over a conventional medium such as ME. The present study is the first to examine the production of lignolytic enzymes using wood-powder-supplemented bran in shallow stationary liquid cultures.

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