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Biobleaching of unbleached and oxygen-bleached hardwood kraft pulp by culture filtrate containing manganese peroxidase and lignin peroxidase from *Phanerochaete chrysosporium*

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Abstract Unbleached and oxygen-bleached hardwood kraft pulp (UKP and OKP), respectively, were bleached with a culture filtrate containing manganese peroxidase (MnP) and lignin peroxidase (LiP) from *Phanerochaete chrysosporium* Burds. The brightness increases of UKP upon biobleaching with the culture filtrate with and without $MnSO_4$ were the same. The brightness increase of OKP with $MnSO_4$ decreased to about half that seen without $MnSO_4$. Changes in the brightness of UKP and OKP by treatment with the culture filtrate were determined. The brightness increased sharply by about eight points during the first 3 h. The 3-h treatment was repeated seven times. The brightness increased linearly with the bleaching of UKP. On bleaching of OKP, the brightness increased slowly and stopped at about 78%.

Key words Biological bleaching · Enzyme bleaching · Lignin peroxidase · Manganese peroxidase · *Phanerochaete chrysosporium*

Introduction

Biobleaching is a process that removes or decolorizes the residual lignin in wood pulp through biological processes. White-rot fungi are the most predominant candidates for biobleaching. So far, *Phanerochaete chrysosporium* Burds.,[1–3] *Coriolus versicolor* (L. ex Fr.) Quel.,[4,5] IZU-154,[6,7] and *Phanerochaete sordida* YK-624[8] have been studied for biobleaching. We previously reported isolation of a new strain, SKB-1152, which can increase the brightness of an oxygen-bleached hardwood kraft pulp (OKP) by more than 80%.[9]

Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are known as extracellular ligninolytic enzymes.[10–12] Recently, it has been reported that MnP was detected during biobleaching by white-rot fungi.[8,13–15] We previously reported detecting LiP activity in residual pulp after washing it with buffer containing detergent during OKP bleaching with SKB-1152. However, we could not extract LiP from the pulp.[16]

Many researchers have tried enzyme bleaching with ligninolytic enzymes. Paice et al.,[13] Kaneko et al.,[14] and Hirai et al.[15] reported biobleaching with MnP. Bourbonnaus and Paice[17] and Call and Mücke[18] reported bleaching with laccase and a mediator. LiP was of little interest.[19,20] In these reports the researchers used purified enzymes, but there is a possibility that some cofactor or mediator would be removed by purification. Furthermore, when enzymes are used industrially, they are used as crude enzymes. Because the crude enzymes contain some other compounds, there is a possibility that they play unexpected roles. Whether the crude enzymes can play the same role as the purified enzymes is not known.

One problem with biobleaching is that it requires a long time. Enzyme bleaching can reduce the treatment time. The treatment times of pulp with ligninolytic enzymes in previous reports[14,20] were as long as 24 h.

In this study we tried to biobleach unbleached and oxygen-bleached hardwood kraft pulp (UKP and OKP, respectively) with a culture filtrate containing both MnP and LiP from *P. chrysosporium*. We also investigated the possibility of short-term treatment (i.e., several hours) and an upper limit of the brightness increase during bleaching.

Materials and methods

Fungi

Phanerochaete chrysosporium (ME-446) was used. This strain was maintained on malt extract agar slants (malt

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extract 3%, yeast extract 0.15%, basal III medium 1%,[21] ammonium tartrate 20 mM, agar 1.5%).

Preparation of culture filtrate

Spores from *P. chrysosporium* were produced on malt extract agar slants, as described above. The spores were placed in a suspension of sterile water and inoculated into stationary cultures at 37°C.[21] The fungus was introduced as a mycelial suspension to 250 ml liquid medium in a 500-ml flask and incubated with royally shaking at 33°C and 150 rpm. The liquid medium was nitrogen-limited. The medium contained (per liter) 10 g glycerol, 0.22 g ammonium tartrate (1.2 mM), 20 mM of sodium acetate, 0.5 g Tween 80, polyurethane foam, and Kirk's trace elements and salts.[22–24] After 0.25 g veratryl alcohol was added to each flask on day 3, the cultures were purged with oxygen every day. After 14 days of incubation, the supernatant was separated from the mycelium by filtration through a glass filter and a membrane filter (pore size 0.45 μm). The filtrate was concentrated to about one-tenth its original volume through ultrafiltration by a MW 10000 cut filter.

Enzyme assay

The enzyme activities of the culture filtrates were measured for LiP, MnP, and laccase. Sometimes the activity of the culture filtrates with added 0.5 mM $MnSO_4$ was measured as well. LiP activity was assayed by monitoring the oxidation of veratryl alcohol (VA) at 310 nm and 37°C.[25] The reaction mixture, a total volume of 2 ml, contained the following compounds and culture filtrates: 0.5 mM VA, 0.5 mM H_2O_2 (final concentration each), 0.4 ml of crude enzyme, and 1.48 ml of 20 mM sodium succinate buffer (pH 3.0). The reference tubes contained the same samples with the exception of substituting H_2O for H_2O_2. These tubes were incubated at 37°C for 5 min. After incubation, the absorbance at 310 nm was measured.

Manganese peroxidase activity was determined by monitoring manganese (III) malonate at 270 nm and 37°C.[13] A 0.2-ml sample was added to a solution containing 0.4 ml of $MnSO_4$ solution (final concentration 0.2 mM) in 50 mM sodium malonate buffer (pH 4.5) and 1.0 ml sodium malonate (50 mM, pH 4.5). The reaction was initiated with 0.4 ml H_2O_2 (final concentration 0.1 mM). The reference tubes contained the same samples with the exception of substituting H_2O for H_2O_2.

Pulp treatments

An unbleached hardwood kraft pulp (brightness 28.9%) and an oxygen-bleached hardwood kraft pulp (brightness 54.0%) produced in an industrial pulp mill were used in this study. Each pulp was well washed and the water removed by centrifugation. A part of each pulp was used in experiments after air-drying (once-dried pulp); the remaining material was not dried (never-dried pulp).

Pulp treatments were done as described below. The reaction mixture's volume was 20 ml and contained 90% (v/v) concentrated culture filtrate, 50 mM glucose, glucose oxidase 0.05 U/ml (final activity) from microorganisms (Oriental Yeast Co.), 0.05% Tween 80, 1% OKP (w/v), and enough 2,2-dimethylsuccinate to adjust the pH to 4.0. In some cases 0.5 mM $MnSO_4$ was added to the reaction mixture. The 100-ml flasks containing the solution were shaken at 140 rpm and 37°C for several hours. After treatment, small handsheets were prepared and their brightness measured by methods used previously.[14] The control was a boiled, inactivated crude enzyme instead of an active crude enzyme.

In some cases the 3-h pulp treatment was repeated. Distilled water was added after each treatment, and the solution was mixed, filtered with a 100-mesh screen, and then bleached again under the same conditions.

Extraction of lignin from pulp with HCl dioxane and infrared analysis

Each pulp sample was refluxed under nitrogen for 2 h with 0.1 M HCl in dioxane/water 82:18 (v/v). The pulp was then filtered and washed with dioxane/water and adjusted with water to a neutral pH. Distilled water was added to the filtrate, which was film-evaporated at 40°C until the dioxane had been removed. The water was never allowed to be reduced so as not to increase the acidity of the solution. After leaving the solution overnight in a refrigerator to support coagulation of the lignin, it was collected by filtration, washed with water to a neutral reaction, and resuspended in water.[26] The extracted lignins were weighed and analyzed by an infrared (IR) spectrophotometer using a KBr method.

Results and discussion

Biobleaching of once-dried and never-dried OKP with culture filtrate with or without the addition of $MnSO_4$

White-rot fungi produce three extracellular ligninolytic enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase.[10–12] When we incubated *P. chrysosporium* in this study, MnP was produced first and then LiP. When both enzyme activities were detected, the cultivation was stopped, and the mycelia were removed by filtration. The filtrate was concentrated to about one-tenth its original volume by ultrafiltration. (The final activities of LiP after treatment with or without $MnSO_4$ and of MnP were 0.144, 0.072, and 1.8 U, respectively). The concentrated filtrate was used as the crude enzyme preparation.

$MnSO_4$ was added to the reaction mixture during the biobleaching with MnP. Here the amount of manganese in the reaction mixture was increased, so we needed to consider the effect of the manganese addition. It is reported that LiP oxidizes manganese at a much faster rate (25 times)

than veratryl alcohol.[27] Therefore, the effect of adding $MnSO_4$ on veratryl alcohol oxidation during biobleaching with the culture filtrate was investigated. These results are shown in Table 1. Both LiP and MnP activities were detected in the crude enzyme preparation. When $MnSO_4$ was added to it, the amount of veratric aldehyde seemed to decrease. Because this crude enzyme preparation contains several compounds (LiP, MnP, VA, $MnSO_4$) several reactions may occur in the mixture. Therefore the results do not always mean inhibition of LiP by $MnSO_4$. Only the decreased VA oxidation is confirmed.

At the beginning of the experiment, two kinds of pulp sample were used: once-dried pulp and never-dried pulp. These pulps were bleached with the crude enzyme preparations without $MnSO_4$. After treatment there was little increase of brightness of the once-dried pulp, whereas the brightness of the never-dried pulp was greatly increased. When bleaching was undertaken with added $MnSO_4$, the brightness increase was not very different (Fig. 1). It was assumed that because fiber bonds were formed upon drying, the enzyme could not gain access between the fibers when once-dried pulp was used. That is, because LiP attacks lignin on the pulp fibers directly, it could not gain access to the fibers when once-dried pulp was used. With never-dried pulp, because fiber bonds were not formed, LiP could gain access to the fibers. Indeed, Srebotnik and Messner reported access of LiP to fibers in never-dried pulp.[28] On the other hand, because the size of Mn(III) as the reacting agent on MnP bleaching is small, it does gain access to the fibers; and in fact MnP from strain SKB-1152 and Mn(III) acetate did bleach once-dried pulp.[14] Accessibility to the pulp fibers is important with enzyme bleaching because the enzyme as the reacting agent is large. Based on these results, never-dried pulp was used as the pulp sample.

Table 1. Changes of absorbance of the concentrated culture filtrate from *P. chrysosporium* with and without manganese sulfate

Culture filtrate	Absorbance (min/ml)	
	ΔA310	ΔA270
Without addition of 0.5 mM of $MnSO_4$	0.38	1.01
With addition of 0.5 mM of $MnSO_4$	0.18	1.01

Note: Laccase activity was not detected.

Biobleaching of UKP and OKP with culture filtrate with or without $MnSO_4$

The OKP and UKP were prepared for biobleaching using crude enzyme preparations. The results are shown in Fig. 2. The brightness of UKP was increased about 10 points after enzyme treatment. The degree of brightness increase of UKP both with and without $MnSO_4$ was not very different. OKP brightness increased about 10 points without $MnSO_4$. There is more brightness increase here than with bleaching by crude and purified MnP from SKB-1152 under the same treatment conditions.[14,29] We think that this result shows the possibility of participation of other factors and enzyme in the crude enzyme preparation during bleaching.

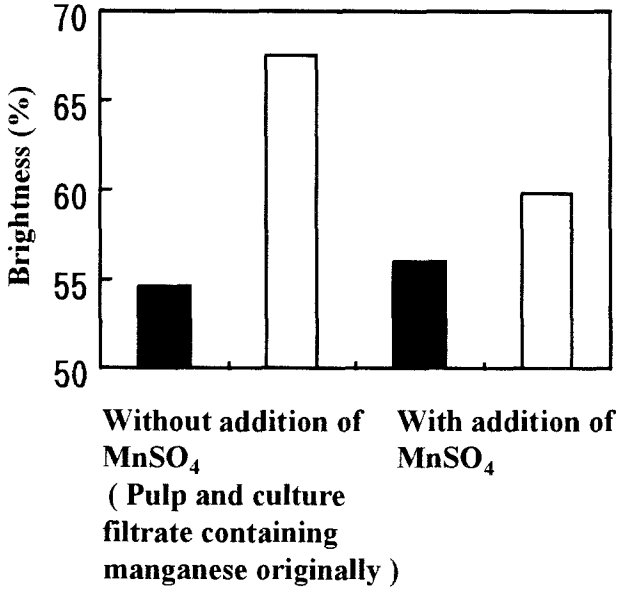

Fig. 1. Brightness of once-dried or never-dried oxygen-bleached hardwood kraft pulp after treatment with culture filtrates of *P. chrysosporium* with or without the addition of manganese sulfate. The original brightness of oxygen-bleached hardwood kraft pulp (OKP) was 54.0%. The reaction mixture's volume was 20 ml and contained 90% (v/v) concentrated culture filtrate, 50 mM glucose, glucose oxidase 0.05 U/ml from microorganisms, 0.05% Tween 80, 1% OKP (w/v), and 2,2-dimethylsuccinate, adjusted to pH 4.0. Some contained 0.5 mM $MnSO_4$. The flasks were shaken at 140 rpm (37°C) for 24 h. After treatment, small handsheets were prepared and their brightness measured. *Filled squares*, once-dried; *open squares*, never-dried

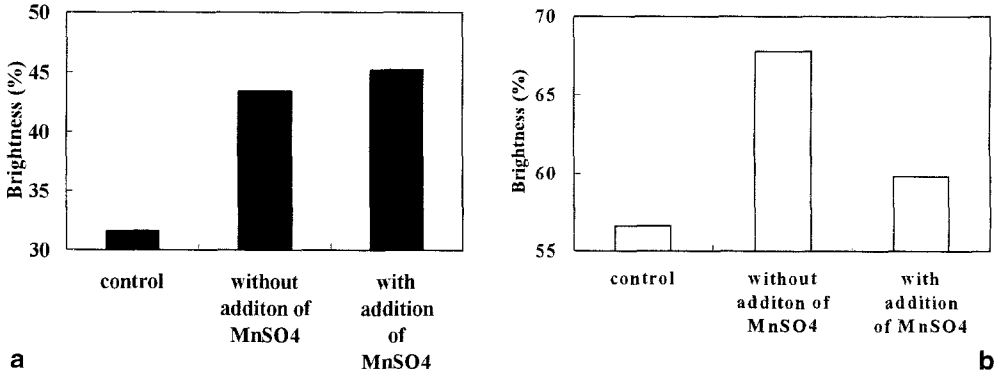

Fig. 2. Brightness increase of **a** unbleached (UKP) and **b** oxygen-bleached (OKP) hardwood kraft pulps after treatment with culture filtrates of *P. chrysosporium* with or without addition of manganese sulfate. The original brightnesses of UKP and OKP were 28.9% and 54.0%, respectively. The controls were subjected to the same conditions as the treatment samples except for containing boiled and inactivated culture filtrate instead of normal culture filtrate

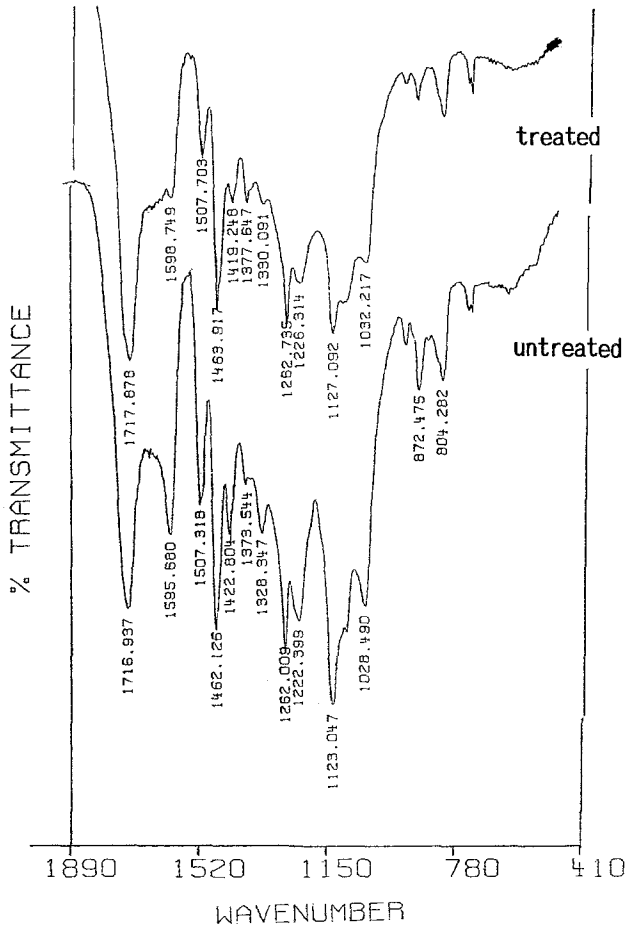

Fig. 3. Infrared spectra of extracted lignins from treated and untreated pulp

We extracted lignin from both untreated and treated OKP without $MnSO_4$ with HCl dioxane,[26] and the extracted lignins were analyzed by an IR method. The amount of extracted lignin from treated OKP was reduced about 40% compared with that from untreated OKP. The IR spectra of each extracted lignin are shown in Fig. 3. The intensities of spectra at 600–1800 cm^{-1} decreased, especially at 1507 cm^{-1} and 1600 cm^{-1} of aromatic skeleton decreased. The relative rate of intensities at 1715 and 1650 cm^{-1} of nonconjugated and conjugated carbonyl skeleton, respectively, increased compared to that at 1500 cm^{-1}. These results suggest degradation of the aromatic skeleton and oxidative degradation of lignin in pulp. On the other hand, only a 3-point increase was observed with $MnSO_4$.

We think this result is related to the LiP reaction and the residual lignin structures in UKP and OKP. The residual lignin in OKP was reported to be less phenolic[30–32] than that in UKP. The degradation of the lignin in OKP would be difficult for Mn^{3+}, which was the activity species produced by the reaction of Mn^{2+} with MnP, LiP, or both. On the other hand, LiP can bleach OKP because LiP can oxidize nonphenolic lignin. If there is much Mn^{2+}, the additional H_2O_2 is consumed in response to the Mn^{2+} oxidation by LiP, and LiP does not react easily with nonphenolic lignin, as noted in Table 1. Mn^{3+} is then produced, but Mn^{3+} hardly oxidizes nonphenolic lignin. As a result, it is supposed that the lignin degradation in OKP may be reduced, and the brightness may not increase sharply if there is much Mn^{2+}. In the case of UKP, we found the brightness increased gradually irrespective of the concentration of Mn^{2+}. We presume this is because there is little nonphenolic lignin in UKP, and Mn^{3+} can oxidize the lignin. Because MnP could bleach UKP, and its activity was the same with or without $MnSO_4$, the brightness increase of UKP after treatment was not so different. On the other hand, because it might be difficult for MnP to bleach OKP and the LiP activity with the addition of $MnSO_4$ decreased, the brightness increase of OKP after treatment with the addition of $MnSO_4$ decreased by about half of that without $MnSO_4$. It was assumed that LiP was not important to UKP bleaching, but it was important to OKP bleaching.

Only about a 5-point increase in UKP brightness by treatment with partially purified LiP has been reported.[20] This result does not agree with our results on the degree of brightness increase. Although we cannot explain the reason for the disagreement exactly, there are four possibilities: (1) The pulp they used might have contained much Mn (the metal concentration in pulp depends on the wood species, pulp washing, and so on). (2) Their LiP activity might not have been optimum. (3) They perhaps removed some cofactor by purification. (4) They might have used once-dried pulp. These points need more detailed investigation with a purified enzyme.

Time course of brightness after bleaching by culture filtrate

The crude enzyme preparation containing LiP and MnP activity could bleach UKP and OKP as described above. Because the brightness increase after treatment without $MnSO_4$ was greater than that with $MnSO_4$ in the OKP treatment and not so different in the UKP treatment, pulp was bleached without adding $MnSO_4$. Figure 4 shows the time course of the brightness change after bleaching UKP and OKP with the crude enzyme. The brightness increased sharply by about seven and eight points for UKP and OKP, respectively, during the first 3 h. This increase during the first 3 h is greater than in a previous report.[20] After that, the degree of increase was reduced with only a slight change after 10 h. When glucose oxidase activity increased to 0.5 U/ml, brightness increased during the first 3 h by nine points (data not shown), but we think that the effeciency of glucose oxidase increase for brightness increase is a little. The brightness increase during the first 3 h showed the potential of enzyme bleaching within a few hours.

Repeated treatment of UKP and OKP with culture filtrate

The 3-h treatments were repeated seven times only. Washings were done between treatments. The results are shown in Fig. 5. The brightness increased linearly upon

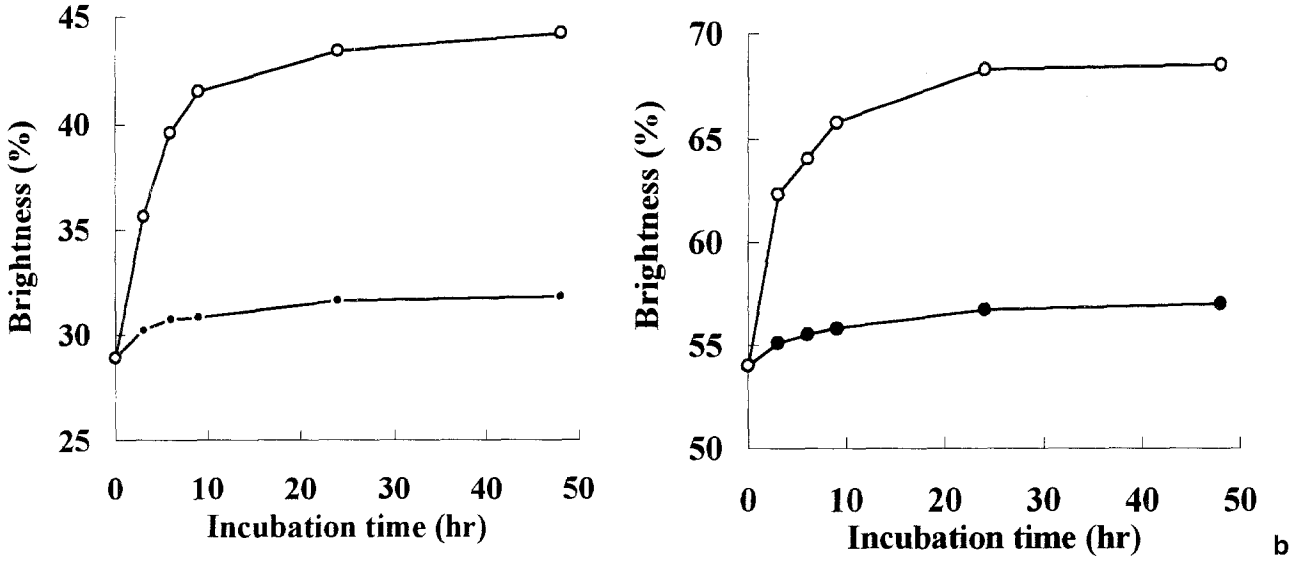

Fig. 4. Changes in brightness of **a** unbleached (*UKP*) and **b** oxygen-bleached (*OKP*) hardwood kraft pulp after treatment with culture filtrate from *P. chrysosporium* with glucose and glucose oxidase. *Open circles*, treatment (culture filtrate, 50 mM glucose, and 0.05 U/ml glucose oxidase); *filled circles*, controls

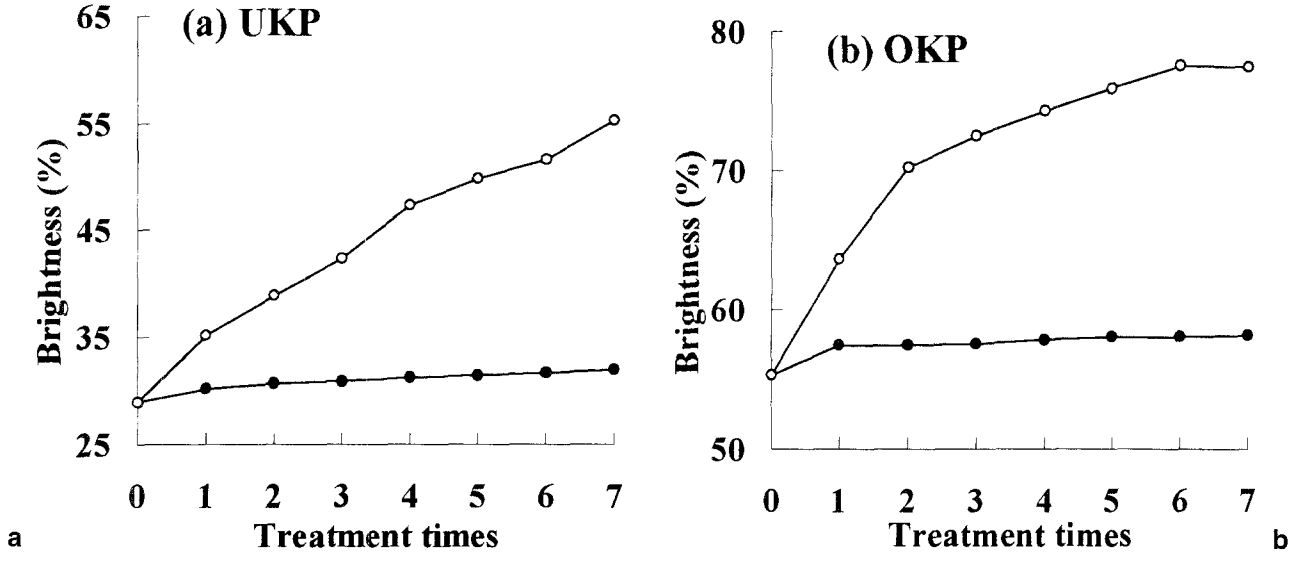

Fig. 5. Repeated treatment of **a** unbleached and **b** oxygen-bleached hardwood kraft pulp with culture filtrate from *P. chrysosporium* with glucose and glucose oxidase. After the first 3-h treatment, distilled water was added, mixed, filtered with 100 mesh screen, and then bleached again under the same conditions. *Open circles*, treatment (culture filtrate, 50 mM glucose, and glucose oxidase 0.05 U/ml; *filled circles*, controls

bleaching UKP. With bleaching of OKP, the brightness also increased sharply after the first and second treatments. After that, it increased slowly and stopped at about 78%. It was assumed that this was the upper limit of brightness increase that could be induced by this crude enzyme. There may be fewer lignin structures in OKP than in UKP that are degradable by LiP and MnP.

Conclusion

The pulps UKP and OKP were bleached by crude enzyme preparation from *Phanerochaete chrysosporium* containing LiP and MnP. The results are as follows.

1. The degree of brightness increase of OKP without the presence of $MnSO_4$ was greater than that with $MnSO_4$.
2. The brightness increase of never-dried pulp was greater than that of once-dried pulp.
3. The brightness increase by the crude enzyme preparation containing not only MnP but LiP and cofactors was greater than that by only MnP, as in our previous report.[14,29]
4. The brightness increased sharply by about seven and eight points for UKP and OKP, respectively, during the first 3 h.
5. The brightness increase of OKP was arrested at about 78% when the 3-h treatment was repeated.

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