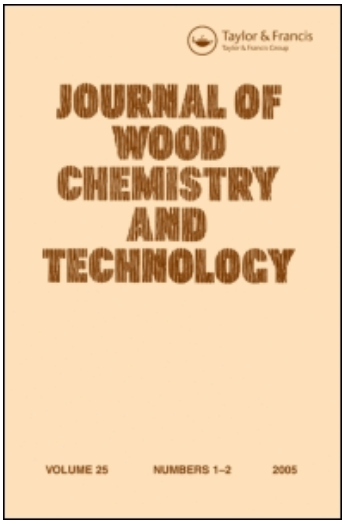

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THE EFFECTS OF TREATMENT WITH THE WHITE-ROT FUNGUS *TRAMETES VERSICOLOR* AND LACCASE ENZYMES ON THE BRIGHTNESS OF DOUGLAS-FIR HEARTWOOD DERIVED THERMOMECHANICAL PULPS

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ABSTRACT

Douglas-fir is a significant west coast tree species that cannot be used as a furnish in the high yield thermomechanical pulping process (TMP) because of the low brightness caused by the high chromophoric extractive content in its heartwood. *Trametes versicolor* and laccase enzymes were assessed for their potential to improve the brightness of Douglas-fir heartwood derived TMP. Treatments with *T.versicolor* were relatively unsuccessful. Although the application of laccase without a mediator decreased the unbleached brightness of heartwood pulps from 40.1 to 34.1 % ISO, after bleaching with H_2O_2, it was found that the laccase treatment increased the bleached brightness from 58 to 61 % ISO. Biological treatments were performed alone and in combination with methanol and alkaline extraction. When pulps were extracted with methanol prior to treatment with laccase and the mediator HBT, the hydrogen peroxide bleached brightness increased from 63 to 64.5 % ISO compared to the non-laccase treated, methanol extracted controls.

INTRODUCTION

Douglas-fir (*Pseudotsuga menziesi*) is a significant tree species on the west coast of Canada constituting 9% of the standing volume and 13% of lumber production in the Province of British Columbia.[1] Douglas-fir possesses excellent structural properties which justify its use in lumber, however its use in pulp production is usually limited to the Kraft process where its dark chromophoric material can be readily dissolved.[2] The dark colour and poor bleachability of Douglas-fir heartwood are thought to be due primarily to the polyphenolic materials derived from the flavonoid dihydroquercetin (DHQ). Based on model compound studies, it has been postulated that DHQ is enzymatically reduced to an unstable leucocyanidin that is subsequently autooxidized to polymeric pigments.[3]

Previously, it was shown that mechanical pulps from Douglas-fir heartwood could only be brightened to 54% ISO, which is a brightness approximately 10-15 pts lower than could be achieved with Douglas-fir sapwood.[4] Similarly, the application of hydrogen peroxide charges of less than 2% resulted in a darkening of mechanical pulps derived from the heartwood. Thus, a cost effective and environmentally friendly method of bleaching Douglas-fir heartwood thermomechanical pulp (TMP) to brightness levels greater than 60% ISO is of interest as it would mean that high grade "wood containing" printing papers could be produced from this type of wood furnish.

There has been no work to date on assessing the potential of biological methods to eliminate the colour problems associated with Douglas-fir TMP. It is known that white-rot fungi possess enzymes capable of degrading all wood components and these enzymes can be used to bleach chemical pulps (biobleaching).[5,6,7] The white-rot fungus *Trametes versicolor*, was shown to be an effective fungus for biological bleaching of kraft pulps, when combined with

alkaline extraction.[7,8] In other work, dihydroquercetin, the compound thought to be responsible for the low brightness of Douglas-fir TMP, has been shown to be modified by *T.versicolor*.[9,10] More recent work, which has attemped to elucidate the biobleaching mechanism of *Trametes versicolor,* has revealed that laccase and manganese peroxidase are the primary enzymes involved.[7,11,12,13] The advantages of direct application of enzymes to the pulps, rather than using fungi, include, eliminating the need for separation of biomass from the treated pulps, decreased treatment times and higher temperature tolerance. It has been shown that laccase production by *T.versicolor* is strongly induced by the addition of dihydroquercetin to the growth media.[9,10] Laccase supplemented with the enzyme mediators ABTS (2,2-azino-bis-6-thiazoline-3-sulfonic acid) or HBT (1-hydroxybenzotriazole) is also able to degrade residual lignin, when followed by alkaline extraction, resulting in the increased brightness of kraft pulps.[14,15,16] The exact role of the mediator compound remains unclear. Laccase is a large enzyme (60-80,000 kDa) which cannot directly access the lignin entrapped within the pulp fibers and it has been shown to mainly oxidize phenolic lignin in the absence of a mediator compound.[17,18] Therefore, it has been postulated that the mediator enhances access to the pulp fibers, prevents repolymerization of lignin radicals produced during oxidation, and allows oxidation of both phenolic and non-phenolic compounds.[19]

Although fungal and laccase treatments of kraft pulps have been shown to be quite effective, treatment of mechanical pulp, particularly those derived from Douglas-fir represent a much more difficult proposition. The high lignin content (25%) of mechanical pulps when compared to kraft (3-5%) would greatly reduce the effectiveness of biological treatment in terms of treatment time and brightness gain achieved.[20,21] However, this same high content of lignin and chromophoric polyphenolic extractives in Douglas-fir mechanical pulp fibers, renders them more

accessible to laccases or manganese peroxidases, when added directly or produced during growth of *T.versicolor* on the pulp.

Previously we found that methanol extraction improved the bleachability of Douglas-fir and western red cedar TMP by selectively removing some of the chromophoric material present in the pulp.[22] In addition, it has been shown that alkaline extraction facilitated biological bleaching of chemical pulps with *T.versicolor* or laccase by removing some of the low molecular weight compounds released during fungal and enzyme trreatment. In the work reported here, we investigated the effectiveness of *T.versicolor* or laccase, with or without mediators in combination with alkali/methanol extraction, in increasing the brightness of Douglas-fir heartwood mechanical pulps.

MATERIALS AND METHODS

Pulp

Non-compression heartwood (NCH) chips from a 129 year old Douglas-fir tree were obtained from the University of British Columbia demonstration forest in Haney, B.C.. Chips were separated and refined to produce non-compression heartwood thermomechanical pulp (TMP) at the Pulp and Paper Research Institute of Canada, Vancouver, B.C..

Laccase Treatments

Samples (8g) of methanol extracted and non-extracted Douglas-fir NCH, TMP were suspended in water at 5% consistency and the pH adjusted to 5.0 with NaOH. Mediators were added along with enzymes to the appropriate samples. Samples were incubated with shaking (200 rpm) for 2 h. at 25°C and boiled for 25 min to deactivate the enzyme. Various samples were alkaline extracted as indicated in Results and Discussion and all samples were bleached with hydrogen peroxide.

Trametes versicolor Treatment

Non-compression heartwood TMP (8g OD) was suspended at 2% consistency and the pH adjusted to 5.0 with NaOH. Mediators were added to the appropriate samples along with the fungal inoculum. Samples were incubated with shaking (200 rpm) for 7 days at 28°C. Appropriate samples were alkaline extracted (see below). All pulps were then bleached with hydrogen peroxide (see below).

Laccase Assays

Laccase was assayed spectrophotometrically according to Bourbonnais and Paice[23] at 420 nm in 0.1 M sodium acetate buffer (pH 5.0) and 0.5 mM ABTS. The oxidation of ABTS was monitored by determining the increase in A_{420} (ϵ_{420}= 3.6×10^4 $M^{-1}cm^{-1}$). One unit (U) was equivalent to 1 μmol of ABTS oxidized per min. Laccase was added to pulp samples at 5 U/g pulp along with 1% ABTS or HBT mediators.

Trametes versicolor

T.versicolor was inoculated onto agar plates (1.5% agar, 0.5% glucose, 0.35% malt extract) and incubated for 7 days at 30°C. Two agar plugs from these plates were used to inoculate glucose peptone media (1% peptone, 2% malt extract, 1% glucose, 0.3% yeast extract) for further incubation at 30°C for 7 days. Twenty mL of culture was homogenized in a Waring Blender for 4 sec and used to inoculate each culture.

Bleaching

All pulp samples were washed with water at 1% consistency and the pH adjusted to 5.5 with NaOH and HCl prior to chelation. Chelation was performed at a 2% consistency with 0.3% EDTA for 30 min at 50°C. After a 1% water wash, pulps were bleached with 8% hydrogen peroxide and 1.5 % NaOH for 3 h at 80°C.

Sodium Silicate (8% $NaSiO_3$) and magnesium sulfate (0.05% $MgSO_4$) were added as peroxide stabilizers.

Methanol Extraction

Pulp (8g OD) was extracted with methanol in a soxhlet extractor for 24 h or until there was no more colour removed. The pulp was subsequently given a 1% water wash and the pH was adjusted to 5.0 with NaOH.

Alkaline Extraction

Pulp (8g OD) was alkaline extracted using 2% NaOH at 60°C for 20 min with stirring. Immediately after alkaline extraction, the pulp was filtered, re-suspended at 1% consistency and the pH was adjusted to 5.0 with HCl.

RESULTS AND DISCUSSION

It is known that *Trametes versicolor* produces laccases and manganese peroxidase when it is grown on Kraft pulps and that these lignolytic enzymes primarily react with the phenolic components of lignin to achieve the observed brightness gain.[7,11,12,13] However, of direct relevance to Douglas-fir pulps, *T.versicolor* has also been shown to modify DHQ.[9, 10] Thus, we first applied *T.versicolor* to Douglas-fir heartwood TMP to try and determine whether this fungus could specifically alter or partially degrade either the lignin or DHQ derived polyphenolic chromophores that are responsible for the low brightness of Douglas-fir heartwood mechanical pulps. Unfortunately, any brightness gain that might have been achieved on the unbleached pulp could not be measured, since the abundant growth of *T.versicolor* interfered with both the handsheet formation and the brightness measurements.

As our previous work had shown[22] that prior methanol extraction of Douglas-fir TMP could remove some of the low molecular weight chromophoric material

present in the pulp, we next compared the effectiveness of *T.versicolor* treatments in combination with prior methanol and subsequent alkaline extraction (Fig. 1a and 1b). As was found in our earlier work with Douglas-fir, methanol extraction of heartwood TMP prior to hydrogen peroxide bleaching was able to increase the final pulp brightness from 59 to 63 % ISO (Fig. 1b). This result differs from Gupta et al where extraction of Douglas-fir heartwood pulp with acetone:water and alcohol:benzene mixtures did not result in any increase in bleached brightness values.[4] However, this discrepancy may be caused by the difference in solvents used for extraction, the age difference of the trees (65 year-old heartwood compared to the 129 year old heartwood used in these experiments), or changes in the chemistry of the wood that occur during refining. These other workers used Douglas-fir RMP[4] whereas Douglas-fir heartwood TMP was used in our study. Alkaline extraction was also performed following fungal treatments since it had previously been reported that alkaline extraction enhances brightness and bleachability by removing the low molecular weight compounds released during fungal treatments of Kraft pulps.[14,24] The increases in brightness achieved by alkaline extraction were comparable to those obtained by methanol extraction (Fig. 1a), while a sequential methanol/alkali extraction resulted in a lower final bleached brightness than when using methanol alone. As there was no cumulative effect when using two different extractions, this implied that methanol and alkaline extraction removed the same substances.

It was apparent that *T.versicolor* (Tv) treatment of heartwood pulp caused the bleached brightness to decrease while fungal treatment of pre-methanol extracted pulps (me-Tv) resulted in a much larger decrease (Figs. 1a. & 1b). It is known that *T.versicolor* produces laccase, lignin peroxidase, and manganese peroxidase enzymes, however it has been shown that only laccase and manganese peroxidase are produced during biobleaching of Kraft pulps.[11,12,25] It is probable that these oxidative lignolytic enzymes are oxidizing the lignin and phenolic extractives

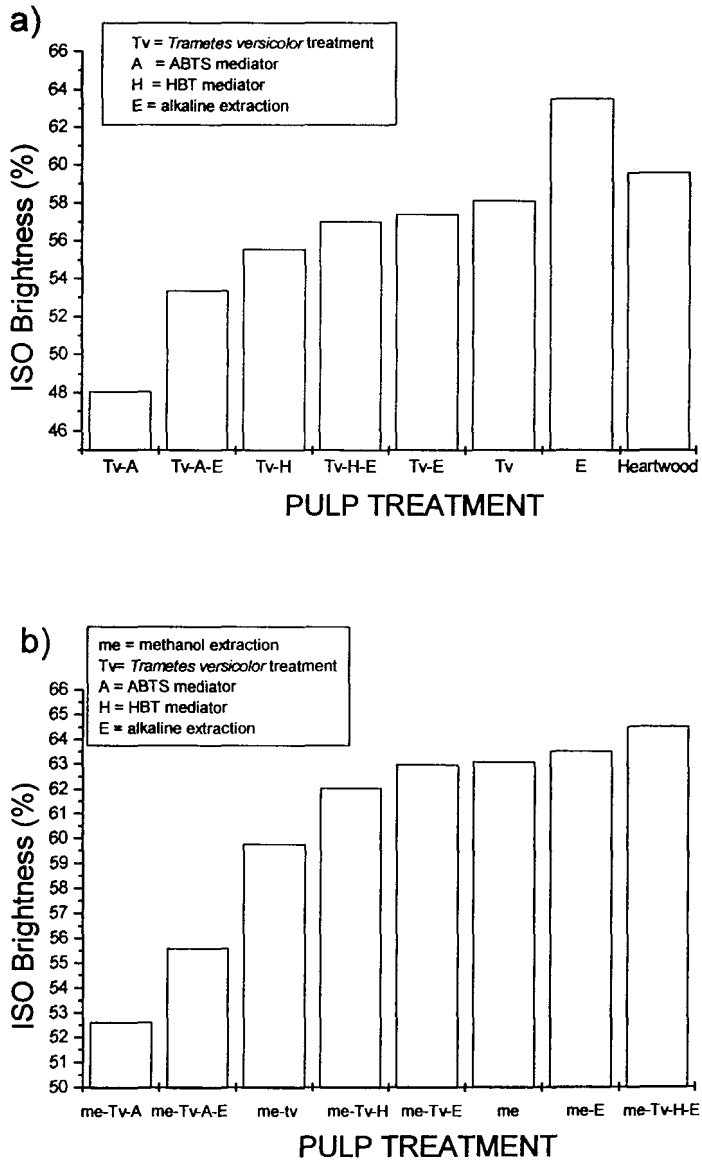

FIGURE 1. Hydrogen peroxide bleached brightness of **a)** unextracted and **b)** methanol extracted Douglas-fir heartwood TMP, treated with *T.versicolor* for 7 days, with and without laccase mediators HBT and ABTS, and subsequent alkaline extraction (treatments are the result of 2 trials performed in the order of symbols shown under each bar)

present in this thermomechanical pulp to coloured quinone and condensed quinone products, resulting in decreased final brightness.[26,27] Alkaline extraction of the sample after *T.versicolor* treatment (Tv-E) resulted in a further 1 pt drop in final brightness, however alkaline extraction after fungal treatment of the methanol extracted pulp (me-Tv-E) restored the final pulp brightness to 63 % ISO. It appears that, once the extractives have been removed, oxidation of the pulp by *T.versicolor* resulted in the formation of a dark, water insoluble, colour that could not be readily bleached. However, much of the colour is soluble in alkali, as alkaline extraction restored much of the pulp bleachability. Apparently, the presence of extractives during *T.versicolor* treatment resulted in the formation of colour more strongly bound to the pulp. It is probable that this is the result of polymerization of the extractives by laccase, analogous to that shown to occur with lignin.[23]

Previous work had shown that the addition of a mediator greatly facilitated the ability of *T.versicolor* derived laccases to degrade residual lignin in Kraft pulps. However, when mediators were added to the Douglas-fir heartwood TMP treatments (Figs. 1a & 1b), the addition of ABTS (Tv-A) decreased final brightness for both the unextracted pulp and the methanol extracted TMP (me-Tv-A). Although alkaline extraction of the pulps prior to peroxide bleaching increased the brightness value to 53 % ISO for the TMP (Tv-A-E) and to 56 % ISO for the methanol extracted pulp (me-Tv-A-E), the final brightness values of these samples were still lower than those of the untreated controls (TMP 58 %, methanol extracted TMP, 63 %). Previously it was shown that ABTS in the presence of laccase results in the formation of a purple colour which is bound to lignin.[14] Since mechanical pulps have a high lignin content that is not removed during peroxide bleaching, this bound coloured complex is probably a significant contributor to the lower brightness that was observed. This contrasts with the laccase/ABTS treatment of Kraft pulps where the lignin along with the colour was

removed during subsequent alkali extraction.[14] The addition of HBT to the pulps also decreased the brightness from for both the unextracted (Tv-H) and the methanol extracted TMP (me-Tv-H) (Fig. 1b). It is probable that an early depletion of HBT during the 7 day treatment leaves the oxidative enzymes produced by *T.versicolor* without a mediator and, as a result, the laccase and other oxidative enzymes may oxidize and/or polymerize lignin and extractives, resulting in a brightness decrease. Subsequent alkaline extraction of the *T.versicolor* and HBT treated pulps recovered the brightness level to 57 % ISO for the TMP (Tv-H-E) and to 64.5 % ISO for the methanol extracted TMP (me-Tv-H-E). With the pre-methanol extracted pulp, the alkaline extraction probably liberated some of the coloured components bound to the pulp, allowing the pulps to be bleached to beyond the 63% ISO level of the control. In the case of the pulp without pre-extraction with methanol, it is possible that polymerized extractives produced during fungal treatment were most likely incompletely removed. Overall, *T.versicolor* treatments of pulps resulted in a decrease in brightness. This was probably due to the oxidation of the large amount of lignin/phenolic extractives present in Douglas-fir TMP, resulting in the formation of coloured products that were not readily bleached by hydrogen peroxide.

In earlier work it was shown that laccase production by *T.versicolor* could be induced by the addition of dihydroquercetin to the growth media.[9] It has also been recognized that laccase is a practical choice for application since, unlike manganese peroxidase, it does not require co-factors such as manganese, chelant and peroxide. Therefore, to try and alleviate some of the problems encountered when using the fungus itself, we next assessed the effects of laccase treatment on the brightness of extracted and unextracted heartwood TMP. As mentioned earlier, in our previous work it was shown that methanol extraction could effectively remove some of the chromophoric material associated with Douglas-fir and western red cedar.[22] As one of the goals of the present study was to assess

the specificity of laccase treatment, with or without the mediators HBT and ABTS, we first compared the brightness values obtained when the unbleached heartwood TMP was extracted with methanol. Although methanol extraction was able to raise the brightness, (Table 1), the laccase treatment drastically reduced brightness. When the pulp was methanol extracted prior to laccase treatment, the brightness was still lower than the untreated control by approximately half of a brightness point. It is probable that this observed decrease in brightness during laccase treatments is due to the oxidation of lignin resulting in the formation of coloured quinone structures.[24,25,26] The addition of mediators did little to alleviate this problem as the largest decrease in brightness, from 40 to 32 % ISO for the unextracted pulp and from 43 to 36 % ISO for the methanol extracted pulp, occurred when the pulp was treated with laccase combined with ABTS. The high a* value of laccase/ABTS treated pulps is indicative of the red component of the distinctive purple colour that was obtained, a result that had previously been observed during the laccase/ABTS treatment of kraft pulps.[14] This earlier work suggested that the purple colour was due to the oxidized ABTS which was covalently bound to the lignin within the pulp. However, with Kraft pulp, the purple colour was readily removed with subsequent alkaline extraction and bleaching steps.[14] As discussed earlier, mechanical pulps possess a significantly higher lignin content which is not substantially reduced during bleaching stages, therefore it is probable that the purple lignin-ABTS complexes would be much harder to remove. Although replacement of ABTS with HBT darkened the pulp to a lesser degree, the a* value of 3.79 (Table 1) resulting from laccase/HBT treatment indicated that a more intense red colour was formed. It was apparent that laccase treatment of Douglas-fir heartwood mechanical pulp with or without mediators did not increase the unbleached brightness.

Although the laccase treatment of pulp resulted in an increase in peroxide bleached brightness from 59 to 62 % ISO (L) (Fig. 2a), no change in brightness

TABLE 1

Unbleached Brightness Values of Douglas-fir TMP Heartwood Pulps Treated with Laccase and the Mediators ABTS and HBT

Pulp Sample	ISO Brightness (%)	L*	a*	b*
Heartwood TMP	40.1	81.41	2.53	19.45
me	43.1	82.14	2.04	19.14
L	34.1	76.72	2.78	20.95
me-L	39.5	79.58	3.09	18.89
L-H	36.2	77.35	3.79	19.15
me-L-H	38.7	79.14	3.16	19.02
L-A	32.2	74.5	4.68	16.91
me-L-A	36.3	78.02	3.30	19.67

(me = methanol extracted, L= laccase treated, H= HBT mediator, A= ABTS mediator)
(Results are the average of duplicates with treatments performed in the order shown)

was obtained through laccase treatment of the pre-methanol extracted pulp (me-L = 63 % ISO) (Fig. 2b). It should be noted that the unbleached brightness of the laccase treated pulp was 34.1 compared to 40.1 for the untreated control (Table 1). Thus, although the laccase treatment decreased the pulp brightness prior to bleaching, it apparently liberated or modified the chromophoric compounds present in the Douglas-fir TMP, allowing it to be bleached to a higher brightness level than the control. The observation that laccase treatment of methanol extracted pulp (me-L) resulted in approximately equal brightness levels to that of the methanol extracted control (me), indicated that the methanolic extractives in the Douglas-fir heartwood TMP were the main site of the reaction for laccase in

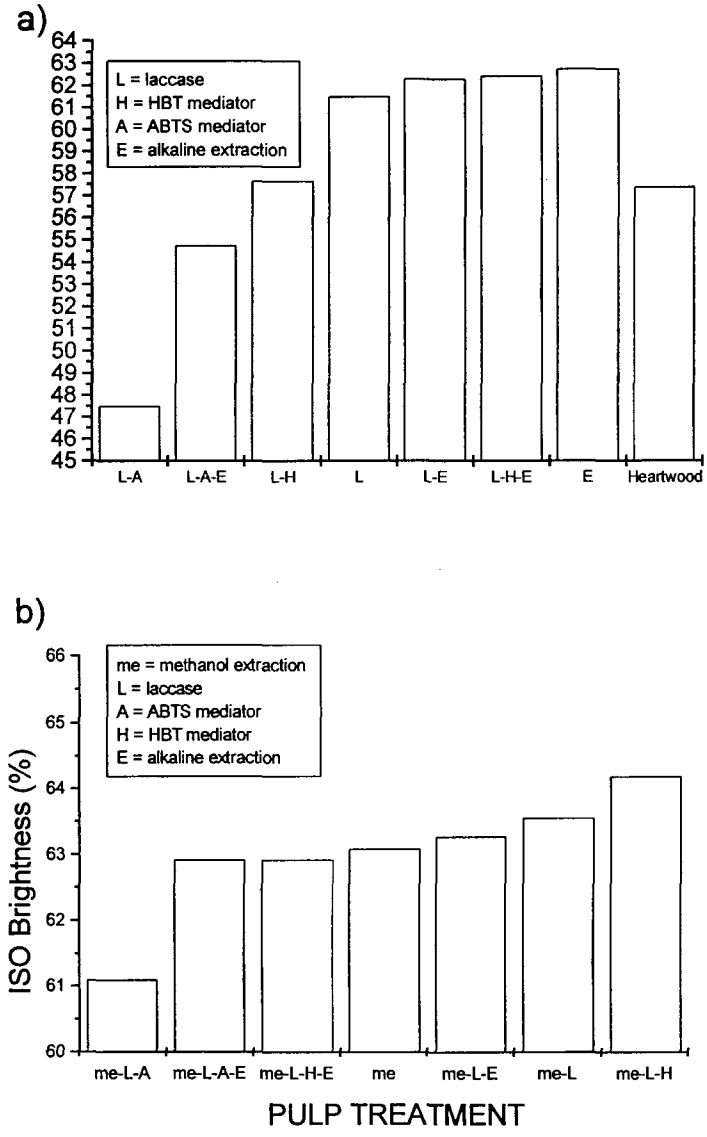

FIGURE 2. Hydrogen peroxide bleached brightness of **a)** unextracted and **b)** methanol extracted Douglas-fir Heartwood TMP, treated with laccase for 7 days, with and without laccase mediators HBT and ABTS, and subsequent alkaline extraction (treatments are the result of 2 trials performed in the order of symbols shown under each bar)

the unextracted pulps. Earlier it was shown that laccases without mediators act specifically upon the phenolic components of lignin.[17] Laccase oxidation of the extractives undoubtedly rendered them more water and alkali soluble, enhancing their removal during and prior to bleaching. Thus the laccases may be modifying the polyphenolic chromophoric extractives increasing their bleachability with hydrogen peroxide.[17] It was apparent that alkaline extraction of the laccase treated pulps increased the brightness of both pulps without methanol extraction (Fig. 2a) as well as methanol extracted pulps (Fig. 2b) which is consistent with the increase in alkaline solubility of the extractives.

Laccase treatment supplemented with ABTS resulted in a significant decrease in brightness for both the unextracted pulp (L-A) and the pulp pre-extracted with methanol (me-L-A). As discussed previously, this is probably caused by the binding of oxidized ABTS to the pulp. Alkaline extraction partially recovered the brightness of the unextracted pulp (L-A-E) but had no effect on the methanol extracted pulps (me-L-A-E). These results are consistent with what was observed with the unbleached pulps (Table 1), again showing that the presence of lignin and methanolic extractives has a profound effect on the binding of the ABTS colour complex to the pulp during laccase treatment.

It was apparent that laccase treatment supplemented with HBT resulted in increased brightness for the methanol extracted pulp (me-L-H) and decreased brightness for the pulp without pre-extraction with methanol (L-H), when compared to untreated controls me-L and L respectively. It is probable that the brightness increase was due to the laccase-HBT combination specifically reacting with the compounds that were not removed during the methanol extraction process, whereas, for the unextracted pulps, laccase-HBT reacted with extractives to create colour and/or bind to the pulp. Although, subsequent alkaline extraction increased the brightness of the unextracted pulp (L-H-E), it decreased the

brightness of the pulps pre-extracted with methanol (me-L-H-E). It appears that the colour formed by the reaction of the laccase-HBT with the extractives is alkali soluble.

Similar to the treatments applied to pulps pre-extracted with methanol, laccase treatments of alkaline extracted pulps were performed in an attempt to determine the effect of laccase on the bound non-extractable chromophoric compounds. Some of the treatments were given a subsequent alkaline extraction after enzyme treatment to remove any alkali soluble colour that the enzyme may have liberated from the pulp, as occurs when biologically bleaching kraft pulps. However, none of the laccase treatments resulted in bleached brightness values beyond that of the alkaline extracted control (Fig. 3). Similar to the results obtained with the pulps pre-extracted with methanol, laccase treatments both with and without mediators had a minimal effect. Overall, laccase treatment of pre-alkaline extracted pulps were ineffective in increasing the bleached brightness values.

CONCLUSIONS

It was hoped that Douglas-fir heartwood derived TMP, which contained significant amounts of polyphenolic material that contribute to the colour of the pulp, would be readily accessible to both the enzymes produced by *T.versicolor* or to the laccases themselves. It was apparent that the fungal treatments were unspecific, affecting a range of target compounds in the pulp. However, laccase enzymes were able to increase the hydrogen peroxide bleached brightness of the methanol extracted pulps when combined with HBT. This implied that the fungal derived enzymes were acting on the methanol insoluble components associated with the pulp. Unlike previous work, laccase treatment of Douglas-fir heartwood TMP, in the absence of a mediator, could enhance brightness. This was probably due to the increased reactivity of the polyphenolic materials with laccase. It

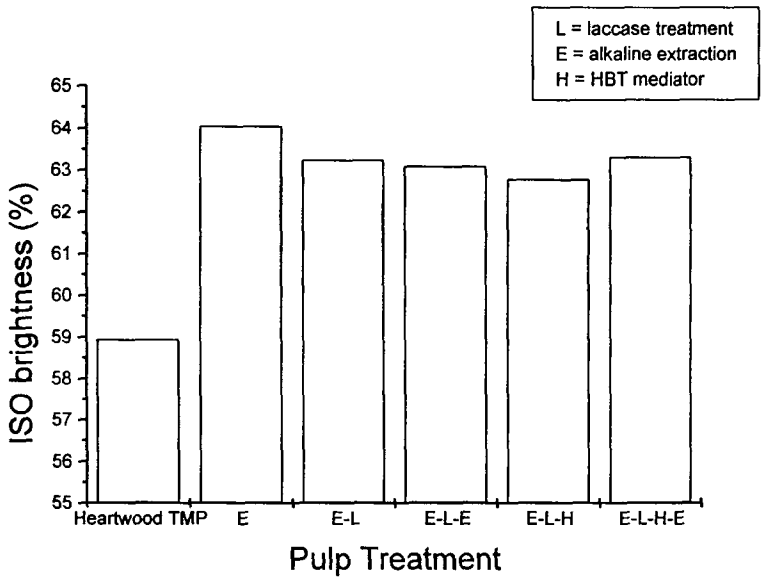

FIGURE 3. Hydrogen peroxide bleached brightness of laccase treated alkaline extracted Douglas-fir heartwood TMP with and without the mediator HBT and a second alkaline extraction prior to hydrogen peroxide bleaching (treatments are the results of 2 trials performed in the order of symbols shown on figure)

appears that laccase treatment was successful because of its primary action on the polyphenolic extractives, while the wider spectrum of redox enzymes produced by *T.versicolor* probably acted on both the lignin and extractives, resulting in the observed darkening of the pulp.

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