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Pulp Bleaching Using Laccase from *Trametes versicolor* Under High Temperature and Alkaline Conditions

M. C. MONTEIRO AND M. E. A. DE CARVALHO*

Departamento de Biotecnologia, Faculdade de Engenharia Química de Lorena, P.O. Box 116, CEP 12600–000, Lorena, SP, Brazil

ABSTRACT

Kraft pulp was delignified using laccase produced by the white rot fungus *Trametes versicolor* immobilized in solid support under specific conditions. The stability tests showed that this enzyme was stable for 6 h at 55°C and pH 8.0, allowing its use under pH and temperature conditions very close to those used in industrial bleaching. In this work, unbleached hardwood Kraft pulp was submitted to prebleaching using 2 U laccase/g pulp basis. Reaction time, temperature, and pH of the enzymatic treatment were investigated. Good results regarding Kappa number reduction, selectivities, and high viscosities were obtained when prebleaching was performed for 1 h at temperature of 55°C and pH 8.0 followed by alkaline extraction and ECF bleaching sequences.

Index Entries: *Trametes versicolor*; laccase; eucalyptus Kraft pulp; biobleaching; enzyme stability.

INTRODUCTION

Conventional Kraft pulp bleaching, employing chlorine and its derivatives to bleach and remove lignin, produces effluents containing a variety of undesirable colored and chlorinated compounds that are often toxic, mutagenic and carcinogenic. Hence, there is a great interest in eliminating or at least reducing the use of these compounds *(1,2)*.

The use of ligninolytic white-rot fungi and their enzymes in biopulping, biobleaching, and effluent treatment has been studied as potential alternative technologies *(3–17)*. Direct biobleaching of pulps by using fungi

* Author to whom all correspondence and reprint requests should be addressed.

is economically troublesome, mostly because of the long treatment time required, but the use of enzymes is promising.

The white-rot fungus *Trametes versicolor* has been studied because of its capability to degrade the three major wood components *(18)*. The fungus can delignify and brighten hardwood Kraft pulp effectively. It also produces the three degrading lignin enzymes, laccase, manganese peroxidase, and lignin peroxidase *(19–22)*. The mechanism of action of these enzymes and their role in lignin breakdown is still under investigation.

Laccase is known to oxidize phenols and phenolic substructures of lignin, and to demethylate phenolic and nonphenolic lignin substructures (23–27). The use of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), together with laccase, can increase the demethylation of pulps during the bleaching *(28,29)*. Its use has been studied by several authors, and, according to Bourbonnais and Paice *(30)*, methanol release and delignification by laccase are reduced in the absence of ABTS.

The objective of this work was to demonstrate that laccase produced under specific conditions can be used in biobleaching of pulp, under pH and temperature values compatible to those used in industrial bleaching.

MATERIAL AND METHODS

Strain Maintenance and Activation

The fungus *T. versicolor* (ATCC 20869) was used for enzyme production. The strain was maintained on 2% malt agar slants. Slopes of the same agar were used for production of spores that were used as inoculum in all fermentations. The concentration of spores was determined using a standard curve that correlated absorbance at 650 nm with spores concentration in terms of number of spores per mL.

Laccase Production

Experiments were performed using immobilized mycelium. For the immobilization step, shake-flask fermentations using a medium containing glucose (2, 5, or 10 g/L), peptone (2 or 10 g/L), trace metals, and 20 m*M* ammonium tartrate buffer at pH 5.0 were carried out in the presence of nylon-web cubes. The 500-mL flasks containing 200 mL of the culture medium were inoculated with 1.0×10^7 spores, incubated at 30°C, and shaken at 200 rpm. Laccase production was also performed in a semicontinuous mode, and, in this case, at defined time intervals, the extracellular medium was replaced by fresh medium.

The carbon/nitrogen (C/N) ratio was calculated, assuming the N content of the peptone used (12%), and the C content of the peptone (60%) and glucose (40%).

Enzyme Assays

Enzyme concentration was determined by measuring the level of activity of the cultivation medium. Laccase activity was determined as de-

scribed by Szklarz *(31)*, with syringaldazine as substrate. The reaction was monitored by change in absorbance at 525 nm (ϵ = 65,000 M^{-1} cm^{-1}) for 1 min. One unit (U) of enzyme activity is the amount of enzyme that oxidizes 1 μmol of the substrate/min. Endoglucanase activity was determined according to Mandels et al. *(32)*, using carboxymethyl cellulose (CMC) as substrate. One U of enzyme activity produces 1 μmol reducing sugars/min.

Enzyme stability

To study the effect of pH and temperature on the stability of the enzyme, a crude enzyme preparation with high laccase activity was separated into samples in which pH was adjusted to the desired values. Then, each one of these samples were divided in 3-mL aliquots and stored at different temperatures. The pHs and temperatures ranged from 5 to 8 and from −10°C to 55°C, respectively. After incubation for the desired time period, the activity was measured according to Szklarz *(31)*.

Analytical

Glucose was determined by the method of Nelson *(33)*.

Pulp Characteristics

The pulp used in the experiments was an unbleached hardwood Kraft pulp (a mixture of various eucalyptus varieties) obtained from Brazilian mills. The initial Kappa number and viscosity values were 15.2 and 28.9 cp., respectively.

Enzymatic Prebleaching

The conditions investigated are listed in Table 1. Both prebleaching and bleaching sequences were performed in polyethylene bags in a water bath at the required time periods and temperatures. Enzymatic treatment assays were followed by a conventional alkaline extraction stage at 60°C for 1 h. After prebleaching, and after each bleaching stage, the pulp was filtered and washed with dH_2O.

Bleaching

The chemical bleaching sequences were used as indicated in Table 2. All concentrations are expressed in terms of air-dried brownstock pulp basis. The DED and DEDED bleaching controls were not submitted to prebleaching conditions; EDED control was submitted to prebleaching conditions, however, without enzyme.

Evaluation of Pulp Treatments

Kappa numbers and viscosities of pulp samples were made according to the standard The Clinical Association of the Pulp and Paper Industry

Table 1
Prebleaching Conditions

Conditions	Temperature 30°C	55°C
Consistency (%)	10	10
Time (h)	1 and 3	1
pH	5 and 8	8
ABTS (m*M*)	0 and 1	0
Laccase (U/g pulp)	2	2

Table 2
ECF Bleaching Conditions

DED sequence	
Conditions:	1° Dioxidation: 1.0% ClO_2, 70°C, 180 min, 6% consistency
	Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
	2° Dioxidation: 0.4% ClO_2, 70°C, 120 min, 10% consistency
DEDED sequence	
Conditions:	1° Dioxidation: 1.0% ClO_2, 70°C, 180 min, 6% consistency
	1° and 2° Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
	2° and 3° Dioxidation: 0.4% ClO_2, 70°C, 120 min, 10% consistency
EDED sequence	
Conditions:	1° Dioxidation: 1.0% ClO_2, 70°C, 180 min, 6% consistency
	1° and 2° Extraction: 2% NaOH, 60°C, 60 min, 10% consistency
	2° Dioxidation: 0.4% ClO_2, 70°C, 120 min, 10% consistency

(TAPPI) methods. Selectivity was defined as the ratio of a desirable bleaching effect (delignification) vs an undesirable bleaching effect (loss of pulp viscosity). Kappa numbers and pulp viscosity values were determined after the first alkaline extraction stage and at the end of the bleaching sequences, respectively. All values reported are means of six replicate experiments.

RESULTS AND DISCUSSION

Laccase Production in Batch Cultures

In order to reach a high laccase level, several glucose and peptone concentrations were tested during the growth phase. When culture media containing 10 g/L peptone and 2 or 5 g/L glucose were used, laccase activity was first detected in 5 d after inoculation, and activity levels of about 800 U/L and 1500 U/L, respectively, were reached in 15 d. When glucose

Fig. 1. Laccase production by immobilized *T. versicolor* using 10 g/L peptone and different glucose concentrations.

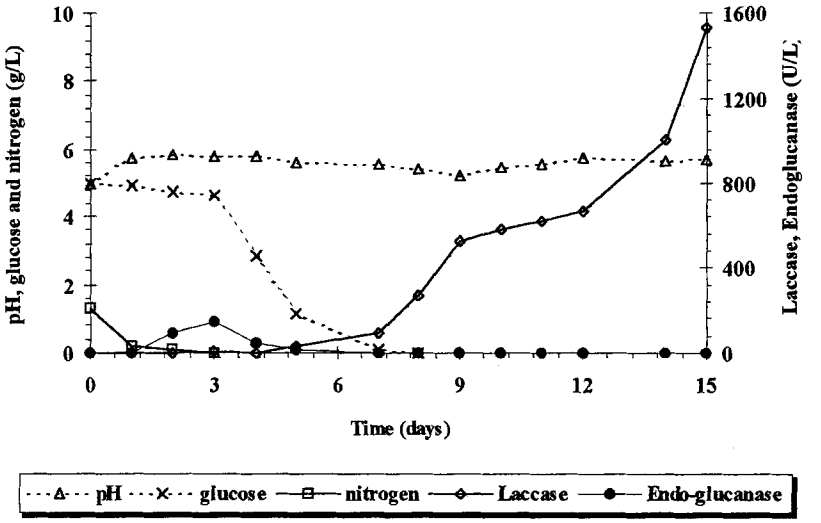

Fig. 2. Laccase production by immobilized *T. versicolor* using 10 g/L peptone and 5 g/L glucose.

concentration was increased to 10 g/L, laccase activity was detected only on d 12, and the activity level was about 500 U/L on d 17 (Fig. 1).

According to the data presented in Fig. 2, which shows enzyme activities, glucose, and N consumption and pH variation against time, using 5 g/L glucose and 10 g/L peptone, the peptone was preferentially consumed. This can be observed once glucose was not consumed until d 3

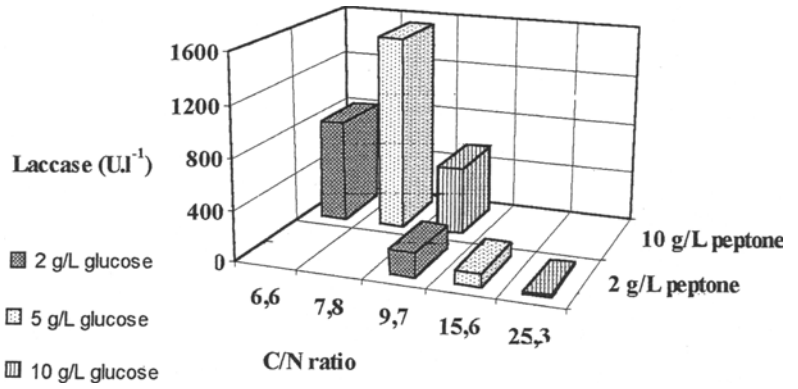

Fig. 3. Effect of carbon/nitrogen ratio on laccase production.

whereas, N from the peptone was used up in 2 d. On the other hand, the pH profile during the fermentation suggested that the buffer was not used by the microorganism as C and N source.

The peptone had an important role in laccase production. The microorganism have mechanisms of metabolism control that are used according to environmental conditions: Medium composition is one of this conditions. This study tested only one source of N (peptone) and two sources of C (peptone and glucose). Figure 3 shows the effect of the initial C/N ratio on laccase production. When 10 g/L peptone was used, high activities were obtained, and the best result was achieved with a C/N ratio value of 7.8. When 2 g/L was used, the increase in the C/N ratio had a negative effect on the amount of enzyme production. Laccase accumulation was affected by C/N ratio, type of substrate (see C/N ratio value of 9.7), and, probably, by the low concentration of N when 2 g/L peptone was used. Thus, other N sources are being tested.

Semicontinuous Laccase Production

The results from repeated batch cultures with immobilized mycelium are shown in Fig. 4. In these experiments, glucose and peptone concentrations were reduced to 2.5 g/L and 5 g/L, respectively, in order to prevent excessive mycelium growth. Under these conditions, it was possible to produce laccase with high activity for at least five successive bath-shake cultures. In this figure are also presented comparative data of enzyme production using immobilized mycelium in shake culture, and free mycelium in stationary culture. The use of bath-shake fermentation made possible an increase in the enzyme production from about 300 U/L to 1500 U/L on a time culture of 15 d. Moreover, the reaction time for successive harvests with approximately the same level of enzyme activity was reduced from 15 to 8 d.

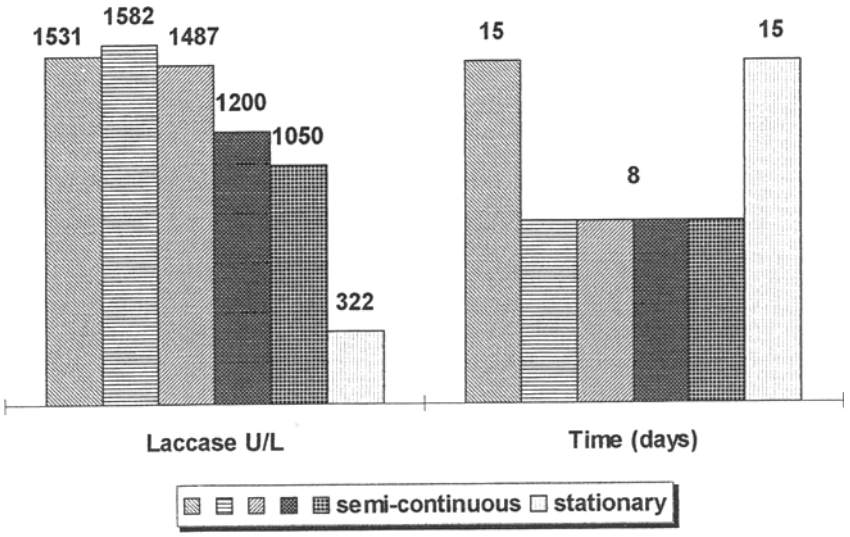

Fig. 4. Laccase production using immobilized mycelium in successive batch-shake cultures and free mycelium in no-agitated cultures.

Enzyme Stability

The enzyme was evaluated regarding storage at different pH and temperature values. As shown in Fig. 5, laccase has good stability at the tested conditions. The best stability was observed in samples maintained under alkaline conditions (pH 8.0) and temperature of −10°C. In this case, after 120 d, laccase lost only 20% of initial activity. A good stability was also obtained at pH 5.7, with a loss of about 30% of the initial activity after 4 mo storage. However, at pH 5.0 a drastic activity decrease (about 40%) was observed in the first days, followed by a very slow decrease in the activity that achieved 50% in 120 d. The enzyme also showed good stability when maintained at temperature of 5°C at all tested pH values.

At temperature of 55°C and pH 8.0, the enzyme was stable for 6 h, with 10% reduction of the initial activity. It was observed that, after 6 h, a significant activity reduction occurred, and, after the 24 h, the enzyme had only 30% of initial activity. However, the maintenance of activity for 6 h was sufficient for laccase action in alkaline conditions and high temperature during biobleaching tests of pulps. This result is interesting, because it points out the potential for the use of the enzyme under industrial conditions.

Bleaching

Several biobleaching experiments were made to evaluate the action of laccase on pulp at different conditions of reaction time, pH, temperature, and the use of ABTS, (*see* Fig. 6). The best results were obtained when

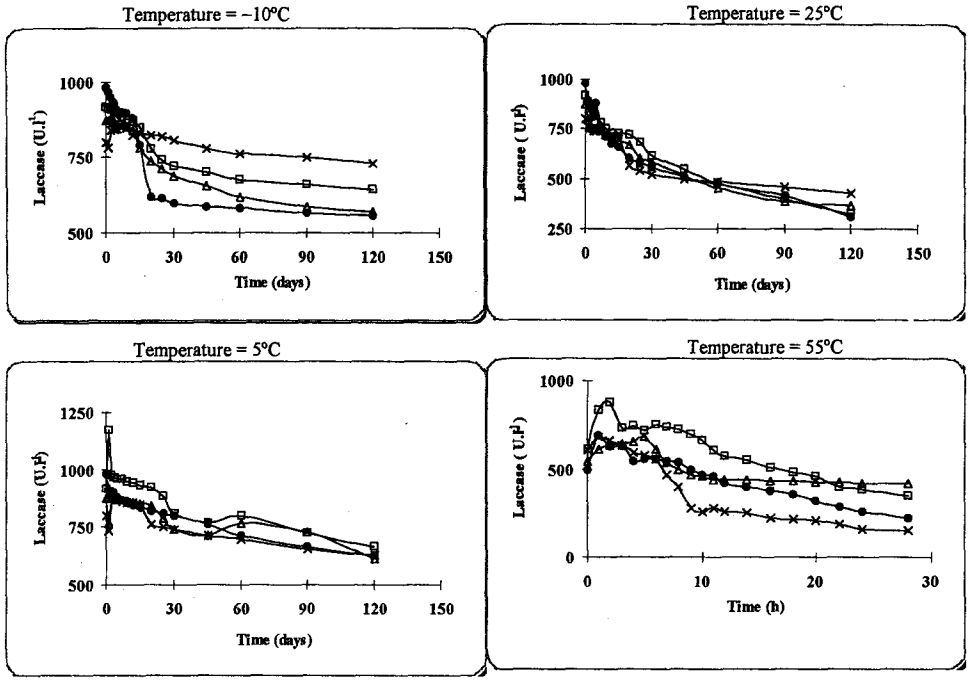

Fig. 5. Laccase stability at different temperatures and pH values. (●) pH 5.0; (■) pH 5.7; (△) pH 7.0; (×) pH 8.0.

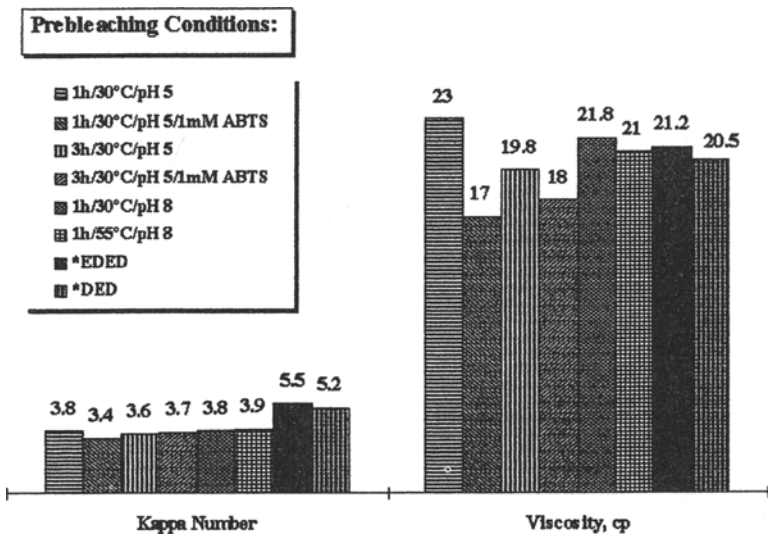

Fig. 6. Biobleaching of hardwood kraft pulp by laccase:
*Bleaching controls were not submitted to the enzymatic delignification. All pulps were prebleached using 2 U laccase/g pulp and submitted to alkaline extraction followed by DED chemical bleaching sequence. Kappa numbers and viscosities were determined after first alkaline extraction-stage (chemical bleaching) and after bleaching, respectively.

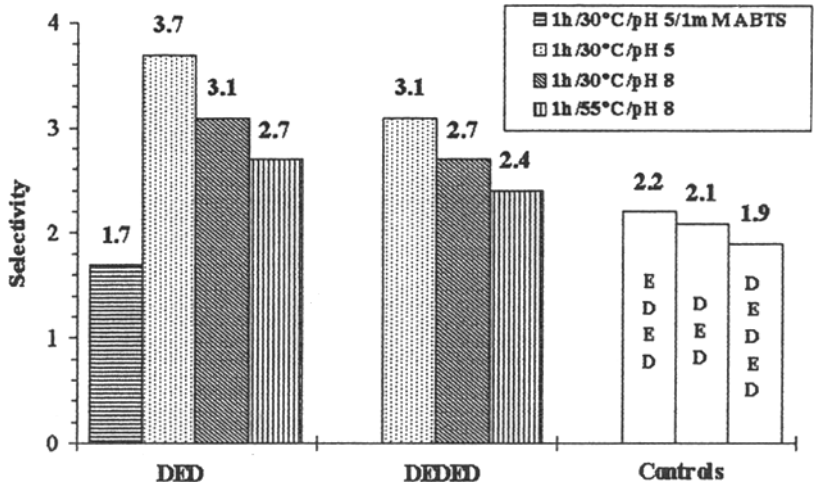

Fig. 7. Selectivity of pulps prebleaching by laccase at acid and alkaline conditions. *Controls were not submitted to the enzymatic delignification. All pulps were prebleached using 2 U laccase/g pulp and submitted to alkaline extraction followed by DED and DEDED chemical bleaching sequences.

pulp was treated for 1 h with laccase at 30°C/pH 5.0, and 30°C/pH 8.0, followed by a subsequent bleaching using a DED sequence. In both cases, the Kappa number was 3.8, corresponding to a 75% delignification and a viscosity reduction of 20 and 24.6%, respectively. Results obtained when pulp was treated for 1 h with laccase at 55°C/pH 8.0 confirmed that laccase can act in alkaline conditions and at high temperature. Kappa number and viscosity were 3.9 and 21 cp, corresponding to delignification and viscosity reduction of around 74 and 27.3%, respectively.

ABTS is frequently used as a mediator to increase the delignification and demethylation during the biobleaching of pulps by laccase *(28,29)*. However, for laccase biobleaching at short reaction times, the use of ABTS yielded a Kappa number reduction greater than that obtained by laccase itself, but viscosities were negatively affected. At reaction times of 1 and 3 h, the use of ABTS increased pulp delignification, resulting in Kappa number values of 3.4 and 3.7, respectively. However, viscosities were affected, and their values decreased to 17 and 18 cp, respectively (Fig. 6).

Figure 7 shows selectivities of pulps treated with laccase, followed by DED and DEDED chemical-bleaching sequences. The best selectivities were obtained when pulps were treated with laccase at 30°C/pH 5.0, followed by both chemical bleaching sequences, DED and DEDED, respectively. The selectivity decreased to 3.1 and 2.7 when the pretreatment was performed at 30°C/pH 8.0, followed by DED and DEDED bleaching sequences. It was also observed that pulps treated with laccase at 55°C/pH 8.0 showed selectivities of 2.7 and 2.4 when submitted to DED and DEDED

sequences, respectively. All these pretreatments resulted in selectivity values higher than selectivity values obtained for the controls.

One unexpected result was obtained when the pulp was treated for 1 h with laccase at 30°C/pH 5.0 in presence of 1 m*M* ABTS. In this case, despite the increase in delignification, the viscosity was not preserved, resulting in selectivity value smaller than those observed for the controls.

CONCLUSION

High laccase activity can be obtained with shake-flask fermentation, in a semicontinuous mode, using a chemically defined medium containing 5 g/L of glucose and 10 g/L of peptone. The laccase produced in these conditions can be stored for a long time at low temperatures. Stability tests showed also that laccase was stable at 55°C and pH 8.0, for sufficient time to allow its use in biobleaching at pH and temperature conditions similar to those used in the pulp industry.

The selectivity depended on the conditions used in both the biobleaching and the chemical bleaching sequences. The best selectivity was showed by pulp pretreated using laccase at 30°C/pH 5.0; however, the use of high temperature and alkaline pH (55°C/pH 8.0) also result in selectivities higher than that obtained for the controls.

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