

EVALUATION OF A NEW LACCASE PRODUCED BY *STREPTOMYCES IPOMOEAE* ON BIOBLEACHING AND AGEING OF KRAFT PULPS

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The aim of this work is to prove the suitability of a new alkaline and halo-tolerant bacterial laccase (SilA) produced by *Streptomyces ipomoeae* CECT 3341 to enhance the conventional chemical bleaching process of an industrial eucalyptus kraft pulp. The laccase used for this study was a recombinant laccase obtained from cultures of *E. coli* BL21 (DE3) grown in LB liquid medium. The biobleaching experiment was carried out on *Eucalyptus globulus* kraft pulps using the above mentioned laccase and acetosyringone as natural mediator. Then, an alkaline extraction and further hydrogen peroxide steps were applied to evaluate the efficiency of the laccase-mediator system as a pretreatment in the bleaching sequences. Biobleached pulps showed a kappa number decrease and a brightness increase without decreasing the viscosity values significantly. Also, a reduction in the consumption of hydrogen peroxide was observed when the enzymatic treatment was applied to the pulp. CIE $L^*a^*b^*$ and CIE L^*C^* color coordinates measured in pulps demonstrated that among all treatments applied to pulps, the laccase-acetosyringone system presented the best optical properties even after an accelerated ageing process. Finally, it is also remarkable that during this treatment 64% of the laccase activity remained unaltered.

Keywords: Biobleaching; Natural mediator; Laccases; *Streptomyces ipomoeae*

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INTRODUCTION

Nowadays, the use of microbial enzymes to treat pulps before applying the standard bleaching sequences is considered to be a valuable alternative to be applied to the pulp and paper industry. These enzymes could help to address the environmental concerns and the low selectivity inherent in the application of the Elemental Chlorine Free (ECF) and Totally Chlorine Free (TCF) bleaching sequences, respectively. With regard to this, the search for ligninolytic enzymes, which could reduce the use of conventional chemical bleaching agents without affecting on pulp quality, is one of the goals for the current research.

Among ligninolytic enzymes, the laccases (EC. 1.10.3.2.) are highly interesting because of their ability to oxidize a wide variety of phenolic compounds (Thurston 1994), requiring only molecular oxygen. Moreover, the oxidative ability of laccases can be extended to non-phenolic structures through the action of some low molecular weight

compounds, called mediators. These mediators, once oxidized by the laccases, become stable radicals that may continue oxidizing other compounds that were not used directly as substrates by the enzyme (Bourbonnais and Paice 1992). The use of both laccases and mediators is commonly known as Laccase-Mediator System (LMS).

Restrictions, such as the high cost of the enzymes and/or the toxicity of synthetic mediators, prevent the industrial full-application of the LMS into the bleaching sequences. In addition, the conditions for enzymatic treatment must fulfil the industrial requirements for a conventional bleaching process. To solve these problems, an intense search for non-toxic mediators and new laccases with specific physico-chemical characteristics for biobleaching purposes is being undertaken.

Regarding the search for suitable laccases to be applied in paper-mills, a novel halo-tolerant and pH versatile laccase (SilA) produced by *Streptomyces ipomoea* CECT 3341 was recently isolated and characterized (Molina Guijarro et al. 2009). The physico-chemical characteristics showed by this enzyme could meet the demands for industrial processes. Thus, the optimal pH for ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) oxidation was 5, which is similar to that described for the laccase of *S. cyaneus* (Arias et al. 2003), and higher to the average value commonly showed by fungal laccases (pH 3). However, the optimum pH for the oxidation of 2,6-DMP (2,6-dimethoxyphenol) was around 8. This alkaline pH activity against a phenolic compound was only described in a few numbers of bacterial and fungal laccases. The unusual resistance of SilA against sodium azide and the high resistance to high salt concentrations maintaining 100 % activity in the presence of 1 M sodium chloride at pH 8 (Molina Guijarro et al. 2009) is also remarkable.

The aim of this work was to evaluate the feasibility of the new laccase produced by *Streptomyces ipomoea* CECT 3341, potentially transferable to an industrial TCF process, to biobleach *Eucalyptus globulus* kraft pulp. For this purpose, delignification degree, hexenuronic acids content, brightness, and CIE $L^*a^*b^*$ color coordinates, were analyzed. In addition, hydrogen peroxide consumption as well as the optical properties stability of the resulted paper sheets were also determined.

EXPERIMENTAL

Pulp

Eucalyptus globulus kraft pulp used for all bleaching experiments was kindly provided by "Factoria La Montañanesa", Grupo Torraspapel (Zaragoza, Spain). The kappa number, brightness, and viscosity of the initial pulp were 14.2, 32.9 %ISO, and 1257 mL g⁻¹, respectively.

Laccase Production and Activity Determination

For this study the recombinant laccase (SilA) of *Streptomyces ipomoea* CECT 3341 was obtained as previously described (Molina Guijarro et al. 2009). For production of SilA, 2 liters of LB liquid medium containing 50 µg mL⁻¹ kanamycin was inoculated with 40 mL of a pre-inoculum of *E. coli* BL21 (DE3) transformed with a pET28 plasmid containing the codifying gene of SilA. After growth, cells were harvested by

centrifugation at 4 °C for 10 min at 12000 × *g*, and the pellet was washed twice with PBS buffer (Sambrook and Russell 2001). To obtain the recombinant protein, cells were suspended in chilled 10 mM phosphate buffer (pH 7) and completely disrupted with a French Press (Sim-Aminco) after two passes at 1500 PSIG. The insoluble fraction was discarded after centrifugation (10 min at 15000 × *g* at 4°C), and the soluble fraction containing the protein was collected. To activate the enzyme, this fraction was incubated for 3 h on ice with 1mM CuSO₄, and then was dialyzed at 4°C overnight against 5 liters of 10 mM phosphate buffer (pH 7), and stored at -20°C until it was used.

Laccase activity was routinely determined at room temperature by estimating the oxidation of 5mM ABTS in 50mM acetate buffer (pH 4.5) monitoring the increase in the absorbance at 436 nm, considering a molar extinction coefficient of 29300 M⁻¹cm⁻¹ for oxidized ABTS (Arias et al. 2003).

Enzymatic Bleaching Stage (LM step)

Assays were performed using 50 g of the industrial *E. globulus* kraft pulp, which was introduced into reactors with the laccase and chemicals. The mixture was intensively mixed before adding oxygen at a pressure of 6 kg per cm², and then the reactors were submerged in a thermostatic bath. Consistency, reaction time, laccase dose, and mediator concentration were fixed during laccase pre-treatment at constant values of 10% (w/v), 1 h, 2.4 Ug⁻¹ oven-dried pulp (odp), and 0.05 mmol/g odp acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone), respectively. These conditions were optimal for laccase activity as previously reported (Eugenio et al. 2010). Temperature and pH were also set at 50°C and pH 8, as these conditions maximize enzymatic specificity against phenolic units of lignin (Molina Guijarro et al. 2009). Moreover, some drops of 0.05 % (v/v) Tween 80 were added in all assays to improve the interaction between enzyme and substrate.

Controls were included in the experimental design as follows:

- i) Conventional bleaching: the enzymatic treatment step omitted
- ii) No-LM: bleaching carried out in the absence of both laccase and mediator, but in the presence of the rest of components used in enzyme treatment at pH 8, 6kg per cm² O₂ and kept at 50°C for 1 h)
- iii) No-LMO₂: bleaching as control No-LM in absence of oxygen, but including the rest of the components
- iv) LM-NoO₂: bleaching as LM step without added oxygen.

In order to evaluate whether laccase activity is inactivated during the enzymatic treatment, laccase activity was measured in each obtained effluent as described above.

Alkaline Extraction and Hydrogen Peroxide Treatments

After the enzymatic pre-treatment, pulps were washed with distilled water until neutral pH and air dried at room temperature. Then they were subjected to an alkaline extraction under the following conditions: 1.5% odp NaOH, 5% (w/v) consistency at 90°C for 120 min. After this treatment, pulps were washed again, and a hydrogen peroxide bleaching stage was applied in the following conditions: 3% odp H₂O₂, 1.5% odp NaOH, 1% odp DTPA, 0.2% odp MgSO₄·7H₂O, and 5% consistency and 90°C for 90 min. Residual hydrogen peroxide was analyzed in the bleaching effluent by standard titration.

Pulp Characterization

Treated pulps were characterized in terms of their kappa number (UNE 57034) and viscosity (UNE 57-039-92). Brightness (UNE 57061) and CIE $L^*a^*b^*$ and CIE L^*C^* coordinates (T 527) were determined using a spectrophotometer ELREPHO 070 (Lorentze and Wettre). Hexenuronic acids (HexA) content was analyzed following the method of Gellerstedt and Li (1996), and its contribution to the pulp kappa number was calculated using the molar oxidation equiv-alent of 8.6. A quantity of 11.6 mol of HexA in 1g of pulp corresponds to approximately 1 kappa number unit (Sevastyanova 2005).

Accelerated Ageing

The bleached and the unbleached pulps were subjected to accelerated ageing to analyze the evolution of their optical properties. This accelerated ageing was carried out in a climatic test cabinet CTS (model C-20/250/S) and consisted in a moist heat treatment at 80°C and 65% relative humidity during 6 days, according to the standard UNE 57092-4. Accelerated ageing pulps were characterized in terms of brightness and CIE $L^*a^*b^*$ and CIE L^*C^* coordinates following the same standards mentioned above.

RESULTS AND DISCUSSION

It is well known that after cooking, eucalyptus kraft pulps contain significant amounts of hexenuronic acids in addition to the residual lignin. These acids behave in a similar way to lignin in the kappa number test, increasing its value (Sevastyanova 2005). They also consume chemicals in pulp bleaching and must be removed to avoid brightness reversion (Forsström et al. 2007). There are other non-lignin substances that also increase the kappa number, but their significance is minor. For this reason, the kappa number is shown in Fig. 1 as the sum of hexenuronic acids and lignin contents.

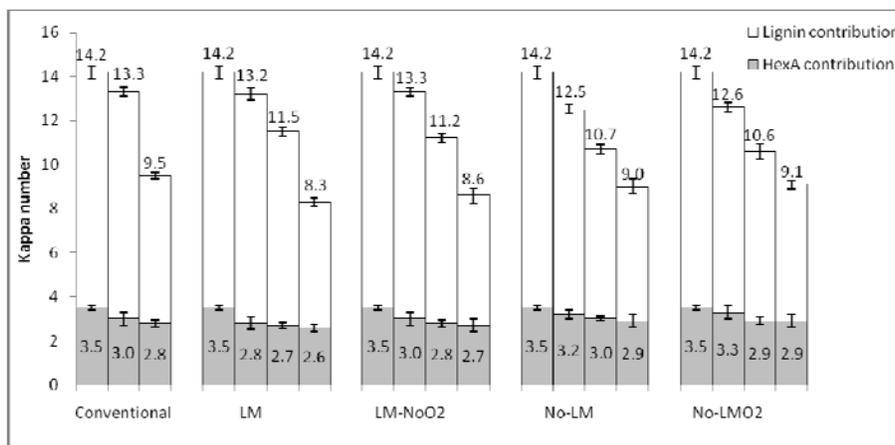


Fig. 1. Kappa number determined as the sum of lignin (white portion) and hexenuronic acids (grey portion) content for each stage in different LM/E/P sequences. Key: Crude = original pulp, E = alkaline extraction, P = hydrogen peroxide, LM = enzymatic pretreatment, LM-NoO₂ = enzymatic pretreatment without oxygen, No-LM = enzymatic pretreatment without laccase and mediator, and No-LMO₂ = enzymatic pretreatment without laccase, mediator and oxygen.

In Fig. 1, a decrease in kappa number can be observed after the application of all LM/E/P sequences. These results are similar to those found by other authors using a fungal laccase (Eugenio et al. 2010). However, it is important to take into account that lower kappa number values were found at the end of the LM/E/P sequences when laccase was used during the pretreatment (LM and LM-No O₂ experiments). After alkaline extraction, it can be also observed that in LM and LM-No O₂ experiments kappa number values were higher than corresponding controls (No-LM and No-LMO₂). However, after the hydrogen peroxide step the lowest kappa number values were obtained when both laccase and acetosyringone were applied (LM and LM-O₂). This decrease was more remarkable in the case of LM treatment, for which a 41.5% reduction was achieved, compared to the 35.9% obtained for No-LMO₂ experiment. This fact can be explained considering that lignin could be modified by the bacterial laccase-acetosyringone system during the enzymatic treatment, making its removal in the subsequent chemical bleaching easier.

As far as oxygen is needed to obtain an efficient enzymatic bleaching process, the comparison of the kappa number values obtained for LM and LM-NoO₂ experiments makes it possible to conclude that the addition of oxygen slightly contributes to the delignification process. This result is not in accordance with those obtained by Eugenio et al. (2010), which indicated that the addition of oxygen during the enzymatic stage increased the kappa number of pulps.

The evolution pattern of the hexenuronic acids content in the proposed bleaching sequences is also shown in Fig. 1, where it is expressed as a contribution to the kappa number. After the conventional E/P stage, the hexenuronic acids content undergoes a global reduction from 3.5 to 2.8 in terms of kappa number contribution (corresponding to 40.6 vs 32.5 μmol/g pulp), because the alkaline stage is responsible for most of the hexenuronic acids removal. This fact is likely due to the extraction of hexenuronic acids bound to xylan–lignin complexes under alkaline conditions (Li et al. 2002; Costa and Colodette 2007). The introduction of a pretreatment with the laccase-mediator system (LM and LM-NoO₂ experiments) in the sequence causes an additional reduction down to 2.6 (30.2 μmol/g pulp). Similar results have been obtained when *E. globulus* kraft pulp was bleached with an industrial laccase and HBT (Valls and Roncero 2009). Finally, it should be noted that hexenuronic acids removal was more remarkable in treatments where laccase-acetosyringone system was present (LM and LM-NO₂ treatments).

In Fig. 2 it can be observed that in most cases brightness increased along the LM/E/P sequence, in agreement with results found by Eugenio et al. (2010). Although a reduction in brightness was observed during the laccase stage (LM and LM-NoO₂ experiments), the further treatment with hydrogen peroxide of these pulps produced an increase in brightness that was higher than in the controls. The increase observed after the laccase stage is not in accordance with the kappa number values estimated for the same experiments, since a kappa number decrease is usually accompanied by an increase in brightness. Notwithstanding, our result was also observed by other authors using similar biobleaching sequences in kraft pulps (Eugenio et al. 2010; Martin-Sampedro et al. 2011), and even an increase of kappa number was reported when *p*-coumaric acid was used as mediator for the biobleaching of eukalyptus kraft pulp with *Pycnoporus cinnabarinus* laccase (Camarero et al. 2007). A possible explanation to support these

results is that during the LM pretreatments the acetosyringone could temporarily remain stuck to the fiber, resulting in a slight decrease in the brightness. On the other hand, our results were similar to those obtained with the laccase of *P. cinnabarinus* and a series of natural mediators for the biobleaching of flax pulp (Fillat et al. 2010).

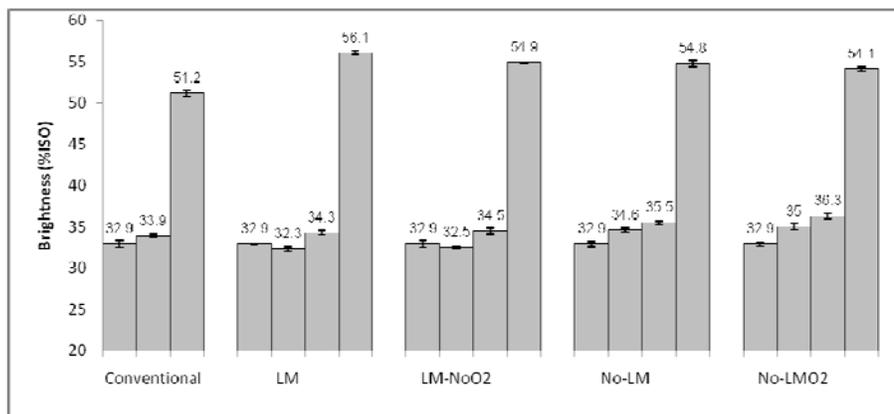


Fig. 2. Brightness (% ISO) corresponding to each stage in different LM/E/P sequences. Key: crude = original pulp, E = alkaline extraction, P = hydrogen peroxide, LM = enzymatic pretreatment, LM-NoO₂ = enzymatic pretreatment without oxygen, No-LM = enzymatic pretreatment without laccase and mediator, and No-LMO₂ = enzymatic pretreatment without laccase, mediator, and oxygen.

All these assays lead to conclude that pulps treated with laccase-mediator system reached the highest brightness (56.07 %ISO) at the end of the LM/E/P sequence. In fact, the brightness of LM pulp was five points higher when compared with those of the conventional bleaching experiments. As it was found for kappa number, the addition of oxygen also improved the brightness. We would like to emphasize that this is the first report that demonstrates the usefulness of a bacterial laccase to delignify eucalyptus kraft pulp at alkaline pH through the action of a natural mediator. The effectiveness of the SilA-acetosyringone system to delignify a hardwood pulp is in accordance with the results obtained in the biobleaching of a soda cook pulp from wheat straw using other alcalophilic bacterial laccase. However, it is important to remark that in this case the pulp was previously treated with a CEH₁H₂ sequence (Singh et al. 2008).

To help judge the importance of our results, it would be interesting to consider the high halotolerance and alcalophilic character of the laccase produced by *Streptomyces ipomoea*, which support the consideration of this enzyme as a good candidate to be used for industrial purposes. These two features could also avoid the additional costs associated with the industrial application of laccases because most such treatments require an acidic and salts-free environment to be effective.

Regarding hydrogen peroxide consumption during the hydrogen peroxide bleaching stage (Table 1), it can be observed that experiments carried out with the laccase-acetosyringone system showed less hydrogen peroxide consumption (85 and 87% for LM and LM-NoO₂ respectively) than the controls (around 95%), besides achieving more delignification. Based on these results it could be inferred that lignin oxidation by the laccase-acetosyringone system facilitates the action of hydrogen peroxide on pulps,

consequently reducing the required amount of this chemical. Thus, while the conventional E/P sequence consumed 94.0% of the initial hydrogen peroxide load, the introduction of the bacterial laccase-acetosyringone system as a pretreatment reduced the consumption to 85% in the most favorable case (LM/E/P sequence). It is known that the consumption of bleaching chemicals is directly related to the content of hexenuronic acids in pulps (Vuorinen et al 1999; Ragnar 2001). This statement could also explain the hydrogen peroxide savings that occurred in the LM experiment, because this pulp contained less hexenuronic acid (Fig. 1). This reduction in chemical consumption contributes to lower amounts of pollutant effluents, lessening one of the main problems associated to the bleaching process in pulp and paper industries.

Table 1. Hydrogen Peroxide Consumption (%) Measured in the Different Bleaching Effluents after the LM/E/P Sequence

Experiments	Hydrogen peroxide consumption (%)
LM/E/P	85
LM-NoO ₂ /E/P	87
Control (conventional E/P)	94
Control (No-LM/E/P)	94.4
Control (No-LMO ₂ /E/P)	95

Brightness (Table 2) and CIE $L^*a^*b^*$ and CIE L^*C^* color coordinates (Fig. 3) were measured in pulps resulting from the P stage before and after the accelerated ageing. These measures were carried out to evaluate the color changes that occurred in pulps during this process. Moreover, these color coordinates were also determined for the unbleached crude pulp.

Table 2. Brightness for all Experiments at the End of the Sequence (LM/E/P) Before and After the Accelerated Ageing

Experiments	Brightness before ageing (% ISO)	Brightness after ageing (% ISO)	Decrease in brightness (% ISO)
LM/E/P	56,07	50,62	5,45
LM-NoO ₂ /E/P	54,91	49,40	5,51
Control (conventional E/P)	51,21	45,61	5,60
Control (No-LM/E/P)	54,84	49,28	5,56
Control (No-LMO ₂ /E/P)	54,13	48,52	5,61

Table 2 shows, as it was expected, a decrease in brightness after accelerated ageing of all samples. Remarkably, the lowest reduction was found for LM/E/P pulp, suggesting that the bacterial enzyme contributed to the preparation of a pulp having more stable optical properties.

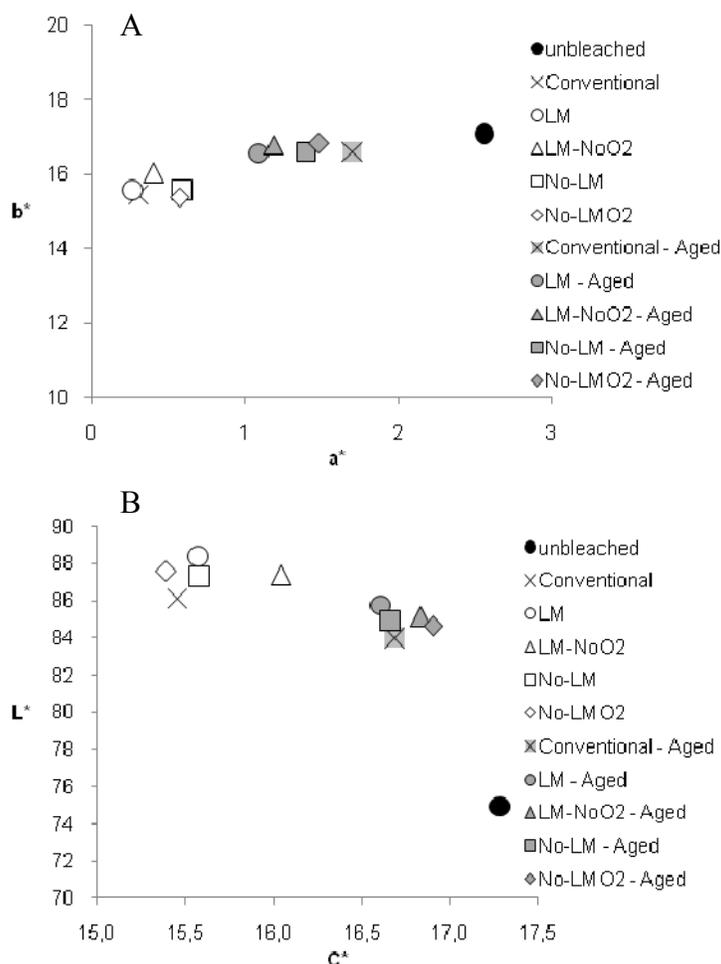


Fig. 3. CIE $L^*a^*b^*$ and CIE L^*C^* color coordinates for all experiments at the end of the sequence (LM/E/P) before and after the accelerated ageing

Figure 3 shows a decrease in a^* coordinate values in all bleached pulps, while the b^* coordinate values were less affected compared with the unbleached crude pulp. However, after the accelerated ageing of these pulps, increases in their a^* coordinate values were evident (grey symbols). It is remarkable that before and after accelerated ageing, the pulps with lowest color (low a^* and b^* coordinates) were achieved when LM treatment was used.

On the other hand, Fig. 3b shows the CIE L^*C^* color coordinates of pulps after the hydrogen peroxide stage before and after accelerated ageing. All bleached pulps increased L^* and decreased C^* (*i.e.* increased lightness and decreased color) compared with the unbleached pulp. Results show that pulp obtained by LM treatment reached the highest L^* value of all bleached pulps. Moreover, this pulp also showed a lower C^* value

than those obtained from the rest of the experiments, apart from No-LMO₂ and conventional treatments. However, after the application to the bleached pulps of the accelerated ageing process, the best CIE L^*C^* coordinates corresponded to the pulp obtained with LM treatment (*i.e.* highest L^* and lowest C^*). By part, the hexenuronic acids present in pulps are responsible for the deterioration of the optical properties of pulps over the course of time (Cadena et al. 2010). Therefore, as far as the LM treatment produces the highest hexenuronic acids removal in pulps (Fig. 1), it could be suggested that the laccase-mediator system used in this study could be considered a good way to preserve the optical properties of kraft pulps against ageing.

Moreover, it is important to point out that a slight decrease in pulp viscosity was found during the course of the LM/E/P sequence for all assays (from 1180 to around 1000 ml/g in all the experiments). The same result was observed by Oudia et al. (2008). These findings demonstrate that high biodelignification can be achieved without decreasing pulp viscosity.

Finally, to support the feasibility of this bacterial laccase for the biobleaching of kraft pulp, it should be noted that just a slight laccase inactivation was detected at the end of the LM treatment (36%). This can be considered a very low ratio of inactivation, as other authors (Moldes and Vidal 2008) described more than 90% of enzyme inactivation after being used in a biobleaching process.

CONCLUSIONS

1. The biobleaching of eucalyptus kraft pulp with the laccase of *Streptomyces ipomoea* CECT 3341 and acetosyringone as mediator produced the most favorable results, not only by reducing the kappa number and increasing the brightness, but also by preserving the CIE $L^*a^*b^*$ and CIE L^*C^* color coordinates after an accelerated ageing process. In any case, a slight reduction of the viscosity of the pulp was observed.
2. The SilA-acetosyringone system used for the treatment of the pulp significantly contributed to the reduction of required chemical amounts, since a significant savings in hydrogen peroxide was achieved at the end of LM/E/P bleaching sequence in comparison with the control.
3. The high percentage of recovery of SilA after the enzymatic treatment of the pulp demonstrated a high stability of this bacterial laccase during the biobleaching process.

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