ENZYMATIC PRETREATMENT TO ENHANCE CHEMICAL BLEACHING OF A KRAFT PULP

PRETRATAMIENTO ENZIMÁTICO PARA MEJORAR EL BLANQUEAMIENTO QUÍMICO DE UNA PULPA KRAFT

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Abstract

Enzymatic xylanases and laccases-rich filtrates were produced by *Aspergillus* sp and *Phanerochaete chrysosporium* ATCC 24725, respectively, using agroindustrial residues as the sole carbon source. Their stabilities on different pH, temperature and ionic salt conditions were evaluated kinetically, and it was also identified the presence of additional enzymatic activities. Finally, an enzymatic pretreatment of kraft pulp was conducted in order to improve the action of chemical compounds used later on. Enzyme filtrates were resistant to the presence of various ionic salts and denaturing solutions, and they had a good stability into a wide range of pH values (from 3 to 9), although they showed low stabilities at temperatures higher than 50°C. Xylanases and laccases were the predominant enzymatic activities observed on the corresponding filtrates. In addition, the enzyme-pretreated pulp had lower kappa number than the not pretreated control. On the other hand, the enzymatic pretreatment produced liquors that showed high contents of residual lignin and chromophore compounds, which indicate the positive action of enzymes on kraft pulp structure.

Keywords: kappa number, xylanases, laccases, *Aspergillus*, *Phanerochaete chrysosporium*.

Resumen

Se obtuvieron filtrados enzimáticos ricos en xilanasas y lacasas producidos por *Aspergillus* sp y *Phanerochaete chrysosporium* ATCC 24725, respectivamente, utilizando residuos agroindustriales como única fuente de carbono. Se estimaron las estabilidades de los filtrados ante diferentes valores de pH, temperatura y la presencia de sales y soluciones desnaturalizantes. Finalmente, se desarrolló con estos un pretratamiento enzimático de pulpa kraft con el objetivo de favorecer la acción posterior de compuestos químicos. Los filtrados enzimáticos resultaron ser resistentes a la presencia de diversas sales iónicas y soluciones desnaturalizantes y mostraron buena estabilidad en un intervalo amplio de valores de pH (de 3 a 9), aunque su estabilidad fue baja en temperaturas mayores a 50°C. Las xilanasas y lacasas fueron las actividades enzimáticas predominantes del filtrado correspondiente. La pulpa kraft que fue pretratada con las enzimas tuvo menor número kappa que la que se sometió solamente a tratamiento químico, y los licores provenientes del pretratamiento enzimático mostraron un mayor contenido de lignina residual y compuestos cromóforos, lo que indica la acción de las enzimas sobre la estructura de la pulpa.

Palabras clave: xilanasas, lacasas, *Aspergillus*, *Phanerochaete chrysosporium*, número kappa.

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1   Introduction

During paper making process, the softening of kraft pulp involves several reactions, in order to hydrolyze cellulose and hemicellulose, and remove lignin. Among paper industries, it is very common the use of peroxides, chlorinated compounds and sodium hydroxide to develop the hydrolytic treatment of different types of wood.   This is known as "bleaching stage" (Georis *et al*, 2000).   Using these chemicals, several toxic residues are generated, such as chlorides and different organic compounds including phenols, terpenes, acidic resines and lignin degradation products.   Some of these products are mutagenic, theratogenic and hardly biodegraded (Valls and Roncero, 2009).   Besides, liquors resulting from chemical treatment of the pulp must be treated to complete mineralization.   This is an expensive process, because the oxidation intermediates tend to be more and more resistant to their complete chemical degradation. Furthermore, they all consume energy (radiation, ozone, etc.) and chemical reagents (catalysts and oxidizers) that increase with treatment time (Oller *et al*, 2011).

The application of enzymes on wood chips or fibers is an attractive alternative to decrease energy demand in the refining process and to introduce novel functional properties on fibers (Torres *et al*, 2012). In addition, the use of enzymatic pre digestion of the pulp could be a good option for diminishing the use of chemical contaminants during pulp kraft stage (Srinivasan and Rele, 1999).   From this approach, several enzymes have been used. Xylanases hydrolyze the hemicelluloses from wood, promoting the removal of lignin, which is trapped in the structure, as well as some derived compounds like the hexenuronic acid (Valls and Roncero, 2009).They are produced by different microbial species, as saprophytic fungi, bacteria and yeasts. Also, the use of some oxidases, like laccases, lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP), produced mainly by white-rot fungi, has been reported on the enzymatic pretreatment of the pulp, as they degrade the lignin structure (Dashtban *et al*, 2010). Biopulping uses the enzymes generated by fungi for reduce the consumption of chemicals in the pulping stage. This increase the yield of fiber, reduces further refining energy requirements, or provides specific fiber modifications (Singh *et al*, 2010; Bertrand *et al*, 2013).

Due to the advantages of the use of enzymes to biopulping process, the need of enzymes with desirable characteristics for a successful inclusion in this process is still present. Furthermore, the need to develop large-scale production and low costs for such enzymes has encouraged the use of economic materials that function as nutrients for the producer organisms. With respect to the special characteristics of enzymes, these must have certain stability at a broad range of temperature and pH conditions, as much as to the presence of several ions and denaturing solutions (Jones and Williams, 2002; Torres *et al*, 2012). However, when an enzyme is used in pulp treatment, it is employed before the chemical process, for obtaining better results (Khandeparkar and Bhosle, 2007).

From the above mentioned, the objective of this work was to characterize two enzymatic rich filtrates, produced by submerged culture on agroindustrial residues, and evaluate the feasibility of using them on the pretreatment of kraft pulp, in order to improve the chemical treatment.

2   Methods

2.1   Microorganisms

For the production of enzymatic filtrates, we used two fungal strains: *P. chrysosporium* ATCC 24725 and *Aspergillus* sp. The last one is a wild strain, with "niger type" characteristics, which was isolated from the raw material area of an important fruit juice making company. Both strains were maintained on Potatoe dextrose agar (PDA) at 4 ºC, by means of periodic reseeds.

2.2   Enzyme filtrates production

We obtained two enzymatic filtrates for the enzymatic pretreatment. One was produced by *P. chrysosporium* ATCC 24725 by solid state fermentation. The other was obtained by means of a submerged culture of a wild strain of *Aspergillus* sp. using 1% (w/v) of wheat bran as the only carbon source in the mineral medium.

The solid state fermentation was developed using sugar cane bagasse as substrate (80% moisture with mineral medium) and 2 mM of $CuSO_4$ as inducer. This culture was grown for 12 days at room temperature (30 °C) and afterwards it was harvested using 100 mM acetate buffer (pH 5.0).

The second culture was grown at 37 °C during 8 days, and the enzymatic filtrate was harvested by filtration. Filtrates were stored in aliquots at the frozen, in which they maintained their activity for several months.

All the experiments developed for obtaining the enzymatic filtrates were done in triplicate, for ensuring the reproducibility of the results.

The composition of the mineral medium (w/v) was as follows: KH_2PO_4, 2.0 g; K_2HPO_4, 2.0 g and $(NH_4)_2SO_4$, 5.0 g on 1L of distilled water.

2.3 Characterization of the enzymatic extracts

Considering the substrates used to develop the cultures mentioned, these were inspected for the presence of several enzymatic activities: endo and exo pectinases (Martínez-Trujillo *et al*, 2009), amylases (Camacho and Aguilar, 2003), CMCases and FPases (Ponce and de la Torre, 1995) and catalases (Fiedurek and Gromada, 2000). Xylanolytic activity was measured by means of quantifying the reducing sugars released after the action of the filtrates on a 1% (w/v) oat spelt xylan solution at pH 5.0 during 20 minutes. One unit of xylanolytic activity was defined as the enzyme needed to release 1 μmol of xylose under the assay conditions (Membrillo-Venegas *et al*, 2013). Laccases were measured after the reaction of the filtrate with a 10 mM guayacol solution at pH 5.0 during 10 minutes at ambient temperature, for which the optical density at 470 nm was registered for estimating the μmol of oxidized guayacol, using the corresponding molecular extinction coefficient ξ_{470nm}= 26600 $M^{-1}cm^{-1}$. One unit of laccase activity was defined as the enzyme necessary to release 1 μmol of oxidized guayacol under the assay conditions (Rodríguez *et al*, 2002).

For thermal stability assays, extracts were incubated at different temperatures among 30 and 60 °C during 8 h, obtaining samples every 2 h, in order to quantify the residual activity.

To monitor pH stabilities, filtrates were diluted in a specific buffer, with the proper pH value, among 3 and 9, and incubated at 37 °C. Residual activity of each filtrate was estimated after 2, 4, 6 and 8 h of incubation under each pH value.

For calculating the inactivation velocity of the enzymatic extracts by the pH and temperature conditions tested, the differential form of a first order inactivation kinetic model was used (Membrillo-Venegas *et al*, 2013). From this, the slope of the enzymatic activity of each filtrate after 8 h of the treatment was calculated.

The effect of the presence of different ionic salts (Mn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}) and chemical solutions (EDTA, Triton, SDS and β-mercaptoethanol) on the enzymatic activity of the extracts was verified too.

For this, we incubate mixtures of equal quantities of enzymatic filtrate and 10 mM of the corresponding salt or chemical solution during 8 h at room temperature, withdrawing samples every 2 h. The residual activity of these samples was estimated.

In all these cases, the residual enzymatic activity (xylanase or laccase) was measured using the analytical technique described above for each enzyme.

2.4 Kraft pulp conditioning

Experimental units were prepared placing into a plastic bag 10 frames of 1 cm^2 of kraft paper and enough water to obtain 8% consistency (1 g of kraft paper for 12 mL of final volume). This mixture was homogenized and allowed to stand during 1 day. Before developing any treatment of the experimental design, these mixtures were submitted to filtration, recovering the corresponding pulp.

2.5 Enzymatic pretreatment of the pulp

Conditioned pulps were submitted to different enzymatic pretreatment, in independent experiments, (their conditions are summarized in Table 1). The final volume of each treatment was 12 mL, for conserving the original consistency of the pulps (8%). Pulps were incubated at 35°C during 14 h, and afterwards were recovered by filtration, in order to be submitted to the posterior chemical treatment. In Control pretreatment (C), pulp was treated with distilled water instead of any enzyme. The resulting liquors of all treatments were analyzed regarding the reducing sugar content and the colorimetric parameters, to know the lignin and degradation subproducts content.

2.6 Chemical treatment of the pulp

Enzymatic and control pretreated pulps were incubated separately with 12 mL of 1.5% (w/v) NaOH during 1 h at 60 °C. Afterwards, pulps were recovered by filtration and submitted to the next chemical treatment, in which 12 mL of 30% (v/v) H_2O_2 diluted in 1.5% (w/v) NaOH were added for incubating the mixture during 2 h at 90°C. Liquors obtained after each chemical treatment were collected separately, to quantify reducing sugar (Miller, 1959), as much as lignin and chromophores compounds content (Bajpai, 2012). The resulting pulp was evaluated using kappa number (TAPPI 236), to estimate the residual lignin. Results obtained were compared with

Table 1. Pretreatments made to the conditioned pulp.

Pretreatment	Operational condition*
Control	Water, at ambient temperature
Xylanases pretreatment	Xylanases (~ 40 U on pH 6.5 citrate-phosphate buffer), at 35°C.
Laccases pretreatment	Laccase (~ 30 U on pH 5.0 acetate buffer), at 30°C
Xylanases and laccases pretreatment	Xylanases (~ 40 U on pH 6.5 citrate-phosphate buffer), at 35°C; and laccase (~ 30 U on pH 4 tartrate buffer), at 30°C

* All the enzymatic pretreatments were developed during 14 h.

Table 2. Enzymatic activities contained in the filtrates.

Enzymatic activity (U/mL)	Filtrate from *P. chrysosporium*	Filtrate from *A. niger*
Xylanases	0.2 ± 0.05	169.0 ± 3.84
Laccases	79.0 ± 1.9	0.0 ± 0
Endopectinases	0.0 ± 0	0.0 ± 0
Amylases	0.0 ± 0	3.22 ± 0.17
CMCases	0.0 ± 0	19.94 ± 0.91
FPAses	0.0 ± 0	28.67 ± 1.4
Catalase	3.0 ± 0.2	3.2 ± 0.14

the initial values, corresponding to the pulp just after the conditioning process, previous to any treatment.

3 Results and discussion

3.1 Enzyme extracts characterization

Considering the nature of experiments by means of which enzymatic filtrates were obtained, these could content different kind of enzymatic activities. All the quantified activities for both filtrates are shown on Table 2.

Phanerochaete chrysosporium was known as a lignin peroxidase (LiP) and manganese peroxidase (MnP) producing white rot fungus. However, the presence of laccase in this specie was demonstrated at the end of the last century. In fact, it has been reported that laccase expression is affected by culture and environmental conditions, mainly the type and concentration of carbon source (Téllez-Téllez *et al*, 2012). At this respect, Dittmer *et al* (1997) showed that *P. chrysosporium* could produce multiple laccase isoforms when CuSO$_4$ was added to the medium, and cellulose was used as carbon source. Also, Podgornik *et al* (2001) detected laccase activity in the semi-solid growth medium of *P. chryssoporium* added with Mn (II) or Mn (IV).

In this work, *P. chrysosporium* was cultivated on sugar cane bagasse, which is a cellulose-rich material, and CuSO$_4$ was added to the medium as inducer. So, we expected that laccases, as well as some hydrolases and other oxidases were present in the filtrate. As can be seen, this filtrate showed catalase and xylanase activities. On the other hand, the filtrates provoked high guayacol oxidation. Considering that oxidases, as manganese peroxidases, could act on guayacol too, it could be difficult to identify if the predominant enzyme of this filtrate was laccase. However, according to Podgornik *et al* (2001), it is known that manganese peroxidases cannot act on substrates as 2-2'-azino-bis(3-etilbenz-tiazolin-6-sulphonate) (ABTS) if catalase is present. At this respect, when we assayed the activity of our extract on ABTS as substrate, we obtained high activities (data not shown). So, we could classify our filtrate as laccase-rich (Table 2).

On the other hand, enzymatic filtrate obtained with *A. niger* is xylanase rich, even when it presents another hydrolytic activities such as amylases and cellulases (endo and exo type). However, these are in low proportion regarding xylanolytic. The low cellulase activity can be seen as an advantage for the use of this filtrate in paper processing.

The stability of the enzymatic extracts at different pH conditions is shown in Table 3. Laccase rich filtrate was relatively stable at the most acidic and alkaline pH

values. This is important, if we consider that during bioconversion processes of lignocelluloses wastes by white rot fungi, many acidic organic chemicals are produced (Mäkelä *et al*, 2002). As laccases contribute to this degradation process, they must be resistant to the acid condition. This could explain the good stability of our laccase rich filtrate, observed at the acid pH values.

A similar behavior was reported by Munusamy *et al* (2008) for two *Pycnoporus sanguineus* laccases, which showed stability on pH values among 3.0 to 5.0, and held lower stability between pH 6 to 9. We also found that one of these enzymes needs to be stored in pH 5.0 to maintain its activity during prolonged incubation times. On the other hand, a halo tolerant-alkaline laccase from *Streptomyces psammoticus* was active in an alkaline pH range, with pH optima at 8.5, and was stable in the pH range 6.5-9.5. Niladevi *et al*, (2008) showed the versatility of microorganisms for producing enzymes with different optimal conditions in order to adapt to the environment in which they are growing. Considering this, the results we obtained could suggest that our *Phanerochaete* strain produces two isoforms of laccases: one acid enzyme and other alkaline. However, further studies need to be carried out to validate strongly this hypothesis.

On the other hand, xylanase rich filtrate was very stable at pH values between 3 and 7, although they lost its stability at the alkaline conditions. Most xylanases known until now are active at acidic or neutral pH. Xylanases from *Streptomyces thermocarboxydus* showed good stability at a pH range from 5 to 6.5 (Kim *et al*, 2010); and activity of purified xylanases from *A. versicolor* was achieved at pH values between 6.0 and 7.0 (Carmona *et al*, 2005). On the contrary, xylanases from *A. fumigatus* were stable at pH range of 3-9

(Thiagarajan *et al*, 2006) and a minor endo-xylanase from *A. niger* seems to be stable at pH values greater than 5.0 (Krisana *et al*, 2005). The purified xylanase from *Thermomyces lanuginosus* was more active in the neutral pH (7.0-7.5), and retained 60% of its activity at pH 9.0 (Li *et al*, 2005).

Considering the inactivation rate values observed for these enzymes (Table 3), it could seem not feasible to use laccase-rich filtrate for developing a pretreatment of the pulp, according to higher values. However, some recent experiments developed in our laboratory showed that it is enough to develop the enzymatic pretreatment of the pulp in a period not longer than 4 h (publication forthcoming), which make feasible the use of our laccase rich filtrate for this process.

Regarding the thermal stability of the enzymatic filtrates (Table 4), laccase activity resulted very sensible to all the temperatures used, especially to the highest. Xylanases activity was more stable, although it diminished considerably at 50°C, and it was definitely lost at higher temperature conditions. This low stability of both filtrates could limit their use in some processes that are developed at high temperatures, such as chemical treatment, in which the wood chips are heating in an aqueous solution of sodium hydroxide (5-20 g/L) and sodium sulfide (10 g/L) from approximately 70°C to cooking temperature (about 170°C) by a 1-2 h (Mejía-Díaz and Rutiaga-Quiñones, 2008). For that reason, we suggest to use both enzymes only as a pretreatment, where one can use an ambient temperature for incubation. In fact, we have identified that it is possible to treat pulps enzymatically using room temperature, obtaining favorable results (Publication forthcoming).

Table 3. Stability of the enzymatic filtrates after incubation during 8 h at different pH values.

pH	Laccase		Xilanasas	
	Inactivation rate (U/h)	Residual Activity (%)	Inactivation rate (U/h)	Residual Activity (%)
3	3.580 ± 1.327[B,C,D]	50.363 ± 1.327[B]	5.412 ± 0.360[B]	82.853 ± 4.143[B]
4	3.028 ± 0.717[C,D]	54.773 ± 0.933[A]	1.629 ± 0.853[C]	88.215 ± 2.931[A,B]
5	4.567 ± 0.962[A,B,C]	41.743 ± 1.923[D]	0.045 ± 0.002[C]	99.094 ± 0.824[A]
6	5.308 ± 1.291[A,B]	23.575 ± 1.326[F]	0.574 ± 0.037[C]	91.471 ± 4.574[A,B]
7	5.709 ± 0.676[A]	29.147 ± 1.457[E]	0.949 ± 0.154[C]	93.763 ± 7.398[A,B]
8	4.343 ± 0.695[A,B,C]	44.253 ± 2.086[C]	9.291 ± 1.488[A]	56.017 ± 2.641[C]
9	3.367 ± 1.205[B,C,D]	52.070 ± 1.941[B]		
LSD*	2.1635	3.0101	1.7576	11.802

* Those treatments with different capital letters have significant differences compared with the corresponding Least Significant Difference (LSD) value.

Table 4. Thermal stability of enzymatic filtrates after incubating during 8 h at the corresponding temperature condition.

Incubation Temperature (°C)	Laccase		Xylanases	
	Inactivation rate (U/h)	Residual Activity (%)	Inactivation rate (U/h)	Residual Activity (%)
30	3.095 ± 0.155^c	68.662 ± 3.433^A	0.071 ± 0.004^C	95.96 ± 5.220^A
40	6.137 ± 0.307^C	37.850 ± 1.893^B	1.371 ± 0.735^C	93.51 ± 3.480^A
50	13.129 ± 0.656^B	33.526 ± 1.676^C	8.649 ± 0.433^B	59.06 ± 2.953^B
60	63.971 ± 3.198^A	27.317 ± 1.366^D	169 ± 0.320^A	0.0^C
LSD*	4.8448	2.8688	1.423	

* Treatments with different capital letter have a significant difference with respect to the corresponding LSD value.

Table 5. Residual activity of the enzymatic extracts after incubation with ionic salts or chemical substances.

Chemical agent (10 mM)	Laccases residual activity (%)*	Xylanases residual activity (%)**
CONTROL	100	100
Metalic ion		
$MnCl_2H_2O$	114.6 ± 23	51.5 ± 4.5
$CaCl_2 2H_2O$	157.5 ± 1.6	37.4 ± 14.5
$FeSO_4 7H_2O$	115.4 ± 33.2	63.7 ± 4.6
$CuSO_4 5H_2O$	162.2 ± 6.7	9.7 ± 10.2
$NiCl_2 6H_2O$	53.4 ± 6.9	20.4 ± 2.5
$HgCl_2$	0 ± 0	0 ± 0
Chemical substances		
EDTA	77.2 ± 6.9	41 ± 0.1
TRITON	113.8 ± 18.7	75.7 ± 10.4
SDS	131.2 ± 14	32.7 ± 7.7
β-Mercaptoetanol	27.9 ± 6.2	58.8 ± 6.1

* 100% of laccases activity is about 79 ± 1.9 U/mL
** 100% of xylanases activity is about 169 ± 3.84 U/mL

The effect of different ionic salts and denaturing solutions on the activity of the filtrates is shown in Table 5. Laccase rich filtrate was affected for the presence of EDTA, a well-known metal chelating agent. This effect could be explained after considering the Cu+2 atoms contained in laccases structure (Ahlawat *et al*, 2007). There are controversial reports about the inhibition of laccases by metal chelators. Some authors have reported that EDTA exerted little inhibition on laccase from fungal origin while in other cases this chelator had no effect on this enzyme, even at a very high concentration (Niladevi *et al*, 2008). However, it is necessary to explain the negative effect of this chelant agent on xylanases rich filtrate. It has been reported that this metal chelator provokes the decrease of xylanase activities produced by several fungi, which indicates that these enzymes could require metal ions for their actions (Kim *et al*, 2010). At this respect, EDTA lead to a 40-50% reduction in enzyme activity of a xylanase from *A.*

niger (Krisana *et al*, 2005); and inhibited the activity of xylanase II from *A. versicolor* (Carmona *et al*, 2005). Besides, a study of characterization of a high molecular weight (about 66 kDa) endo-xylanase from *Aspergillus fumigatus* reported that EDTA were a moderate inhibitor of xylanase activity, because it is an amino acid modifying reagent (Thiagarajan *et al*, 2006). It could be one of the causes of the actions of this chelator on our xylanolytic enzymes.

Some ionic salts, as Mn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+}, that could be present in the kraft pulp (Torres *et al*, 2005), did not show any negative effect on laccases rich filtrates, but it were adverse for xylanolytic activities. Hwang *et al* (2010) suggest that the inhibitory effect of metal ions on xylanase activity implies that this enzyme might have a metal binding site near the catalytic region that were inhibited at different levels by various metal ions. Mercury was adverse for both enzymatic activities. Broadly, laccases filtrate showed to be more stable or enhanced

with the ionic salts used.

It would be important to have a further characterization of both filtrates, for a better understanding on their effect on kraft pulp. At this respect, we consider that an SDS-PAGE with the corresponding zymogram could proportionate more information about enzymes that compose these filtrates. So, for the application of some of them, it would be feasible to purify them partially or totally in order to obtain its best performance during the process. However, at this stage we only wanted to prove how the effect of applying these filtrates in the pretreatment of kraft pulp was and in that way, identify the feasibility of use them for contributing to diminish the chemical charge of the process. A future work must consider, for sure, a most detailed characterization of the filtrates.

3.2 Enzymatic pretreatments

Biological bleaching of pulp has been approached mainly by the use of lignolytic and hemicellulolytic enzymes. At this respect, xylanases remove the lignin-carbohydrate complex that act as physical barriers to the entry of bleaching chemicals, and then the lignin layer is easily available for penetration and degradative action of laccases (Kapoor *et al*, 2007). So, enzyme treatment of wood chips can open up the cell wall structure and, hence, lead to fiber separation at preferable locations in subsequent refining (Torres *et al*, 2012).

As simultaneous action of xylanases and laccases may have a tremendous potential for bleaching of paper pulp (Dwivedi *et al*, 2010), we used our enzymatic filtrates for developing different pretreatment to a kraft pulp, in order to identify the feasibility of their use for improving the chemical treatment of this pulp. For doing this, we developed different treatments, in which the corresponding enzymatic pretreatments were developed during 14 h to the conditioned pulp, and the chemical treatment was developed immediately after that. For the control, the chemical treatment was applied to a pulp pretreated only with tapping water. Results obtained are summarized in Table 6.

With respect to pulp properties, we decided to measure Kappa number, in order to estimate the lignin and hexenuronic acid content after each treatment. Results from all the experiments showed that kappa number of pulp diminished with respect to its initial value (26.15 ± 1.31). Samples that were pretreated with only one enzymatic activity showed values

similar to those for pulps submitted to a chemical treatment without enzymatic pretreatment (named Q on Table 6). The only significant diminution of kappa number (around 32.1 %) was obtained when both enzymes were used in a sequential way. Similar results were reported for eucalyptus, mixed wood and soda-antraquinone pulp from oil palm empty bunches, in which a pretreatment with xylanase-laccase sequence provoked a considerable diminution of kappa number and higher brightness values than pulp samples without enzymatic pretreatment (Valls *et al*, 2012; Dwivedi *et al*, 2010; Martín-Sampedro *et al*, 2012).

On the other hand, when both enzymes were applied sequentially to kraft pulp before the chemical treatment, the highest weight lost was around 22%. This result indicates that enzymatic pretreatment maintained the yield of the kraft pulp. Recently, in our laboratory we obtained good biopulping results using only enzymes and, in this case, the efficiency of the process was really high. We obtained at most 12% of weight loss in the treated material, while that treated chemically lost around 75%.

It is important to highlight that our results were as good as those reported for systems in which a mediator was used for improve the action of a commercial laccase (Valls *et al*, 2010). So, by using fungal xylanases and laccases, in this work we reached a kappa number reduction of about 8 units, which is slightly higher than the decrease reported for kappa number of a *Eucalyptus globulus* pulp using two different mediators (Eugenio *et al*, 2010).

Summarizing, even when in this work we did not evaluate the brightness and the viscosity of our samples, our results suggest that the industrial implementation of sequences based on xylanases and laccases pretreatment is feasible.

Regarding the liquors generated after the different treatments, some significant differences were observed too, as can be seen on Table 6. Optical density at 280 nm indicates the presence of lignin in the released colored compounds after each treatment (Khandeparkar and Bhosle, 2007). At this respect, results from enzyme pretreatments were about 10 fold higher than those coming from the pure chemical treatment, even when in both cases the kappa number showed a diminution with respect to the control experiment. This suggests that chemical treatment acted on pulp, degrading the lignin and not only freeing it from its structure. In fact, it is known that approximately 90% of the lignin and 30% of wood carbohydrates are dissolved during chemical digestion

Table 6. Pulp characteristics after different treatments

Treatment	Kappa number*
Initial value, pulp just before conditioning process	26.15 ± 1.31^A
Q	20.56 ± 1.1^B
X	20.62 ± 0.57^B
L	20.56 ± 0.52^B
XL	17.76 ± 2.061^C
LSD	1.4574

* Values with different capital letter had a significant difference ($p<0.05$) with respect to the corresponding LSD value.

(Bajpai, 2004). On the other hand, the function of enzymes during pretreatment phase could consist in release the lignin of the pulp structure and probably degrade it partially, facilitating the posterior work of chemical substances.

In fact, it is well known that the main effect of enzymatic treatments is that wood chips become softened and more porous, making them more likely to broke during pulping (Torres *et al*, 2012). Besides, xylanases are considered as bleach boosters, because a limited hydrolysis of the xylan network is often sufficient to facilitate and enhance the subsequent chemical attack on lignin with various bleaching chemicals (Bajpai, 2004; Valls *et al*, 2010). This was proved on a work, were authors reported that xylanase pretreatment of the pulp facilitated the access to cellulose fibres, thereby boosting the effect of the laccase-mediator system in reducing the content of residual lignin and releasing more hexenuronic acids (Valls and Roncero, 2009).

Chromophore groups as much as some hydrophobic compounds detected in the liquors by registering the optical density at 480 nm (Khandeparkar and Bhosle, 2007), increased considerably with all enzymatic pretreatments (Table 6). This increase was higher when laccases were used. This suggested that the dissociation of lignin-carbohydrate complex (LCC) from the pulp could be happening, as was reported before in a similar process using only xylanolytic activity during kraft pulp biobleaching (Khandeparkar and Bhosle, 2007).

During chemical treatment, NaOH acts directly on lignin and the polysaccharides of kraft pulp structure, hydrolyzing them to the constitutive reducing sugar (Hubbe and Bowden, 2009). However, the reducing sugar content of liquors obtained after the chemical treatment was lower than those in which an enzymatic pretreatment were developed (Table 7). In this last case, the highest reducing sugar content was observed on those experiments in which only one enzyme was used for pretreatment phase. But when both enzymes were used consecutively, this content was reduced more than a half. Even though, reducing sugar content increased with the enzymatic pretreatment of the pulp.

The release of reducing sugars, lignin and phenolic compounds are interrelated phenomena. When kraft pulp was pretreated only with xylanase, the xylose and other reducing sugars would be released from the hemicellulose layer, resulting in an increase in the free sugar content, as well as lignin and phenolic compounds from the pulp fibers, which ultimately caused the enhancement in absorbance of the resulting liquors compared to the control. Besides, in those cases in which laccases were used as the only enzymatic pretreatment, these enzymes could be acting on the upper lignin, stimulating the attack of polysaccharides like xylan during the posterior chemical treatment developed with NaOH. This behavior had been reported before for the action of xylanase from *Streptomyces* sp. QG-11-3 on eucalyptus kraft pulp, in which a scanning electron microscopic study revealed that enzymatic pretreatment introduced greater porosity, swelling up and separation of pulp micro fibrils and pulp fibers, which renders fibers more susceptible to the chemical process (Beg *et al*, 2000).

On the other hand, the pulping process generates a considerable amount of wastewater, most of which is too weak to be recovered, even when it is highly polluting (Thompson *et al*, 2001). In general, BOD of liquors produced during pulp and paper making process is around 1.5 g/L, and the suspended solids are around 3.29 g/L (Pokhrel and Viraraghavan, 2004). Both values are higher than those established by the Mexican official norm (NOM-001-ECOL-1996) for being released as effluents, which is around 200 mg/L. Therefore, these liquors must be stabilized before being released to municipal drain, especially those from chemical treatment, whose pH is about 10. However, their high solids content and BOD values could be due to the presence of high quantities of reducing sugars. This makes liquors valuable for being used as a culture medium for the growth of some microorganisms without the addition of extra nutrients, only by adjusting the pH. Besides, during their growth, these microorganisms can produce some enzymes that can be industrially significant, as laccases.

Table 7. Liquor characteristics after different treatment of kraft pulp

Treatment	O.D. 280 nm	O.D. 480 nm	Reducing sugar content (g/g of pulp)
C	17.04 ± 0.11^{B}	0.82 ± 0.06^{D}	0.115 ± 0.006^{D}
Q	8.66 ± 0.56^{B}	0.44 ± 0.09^{D}	0.228 ± 0.011^{C}
X	177.3 ± 10.4^{A}	3.63 ± 0.61^{C}	1.32 ± 0.066^{B}
L	177.4 ± 5.4^{A}	4.7 ± 0.35^{B}	1.4 ± 0.07^{B}
XL	186.3 ± 16.8^{A}	3.93 ± 0.35^{C}	0.58 ± 0.029^{A}
LSD*	20.298	0.5093	0.0613

* Those treatments with different capital letters have significant differences compared with the corresponding Least Significant Difference (LSD) value.

Conclusions

The laccase and xylanase rich extracts produced by our fungal strains, showed good characteristics for being used in the pretreatment of kraft pulp. So, with the enzymatic pretreatment applied sequentially previous to the chemical, we obtained a decrease of 11% in kappa number compared to that obtained with a pure chemical one, without losing more than 10% of the original pulp weight. The analysis of chromophores and reducing sugar on liquors obtained after every treatment showed a considerably increase, which suggested that enzymes act on pulp structure, facilitating the posterior chemical action. So, the use of enzymes could help to reduce the use of some chemical, with the concomitant reducing in the ambient impact.

Acknowledgements

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