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Black Liquor Decolorization by Selected White-Rot Fungi

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Abstract Five different strains of white-rot fungi have been tested for their ability to decolorize black liquor on plates and on solid-state fermentation using vermiculite as the solid inert support. Since the high salt concentration inhibited the growth of all fungi, the black liquor was dialyzed against distilled water prior to use. A preliminary step on plates was carried out to qualitatively determine the capacity of the fungal strains for black liquor decolorization. Out of the five fungi studied, *Phanerochaete sordida*, *Pycnoporus sanguineus*, and *Trametes elegans* exhibited the more conspicuous decolorization halos in malt extract medium, while the decolorization by all the strains was not evident when a defined culture medium was used. Cultures on solid-state fermentation using vermiculite as solid support were also tested, the liquid phase was malt extract or glucose-based medium and supplemented with different black liquor concentrations. Decolorization of black liquor was largely affected by the fungal strain, the concentration of black liquor, and the carbon source. The percentage of color removal ranged from 6.14% to 91.86% depending on the fungal strain and culture conditions. Maximal decolorization was observed in malt extract cultures after 60 cultivation days. Interestingly, decolorization in malt extract medium increased with increasing black liquor concentration. The highest decolorization value was achieved by *Steccherinum* sp. which reduced up to 91.86% the color of the black liquor in malt extract medium; this percentage is equivalent to 5.2 g L^{-1} of decolorized black liquor, the highest value reported to date. Traditional technologies used for the treatment of black liquor are not always effective and may not to be an environmentally friendly process. Vermiculite–white-rot fungi systems are presented in this work as a promising efficient alternative for the treatment of black liquor.

Keywords Black liquor · Decolorization · Laccase · MnP · White-rot fungi

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Introduction

Black liquor is a residue from the most widely used process in the industrial production of cellulosic pulps, kraft pulping. Wood and non-wood lignocellulosic materials are treated at high temperatures (150–170 °C) with an aqueous alkaline solution composed essentially of sodium hydroxide and sodium sulfide. All of these treated materials have cellulose and lignin as main components. Lignin, an aromatic polymer composed of dehydropolymerized structural units derived from phenylpropane and primarily responsible for the cohesion between fibers in wood tissues, is degraded and dissolved almost completely (90–95%). This aqueous solution containing the inorganic and organic reaction by-products constitutes the black liquor. During pulping treatments, chromophoric and highly oxidized polymeric lignin/chlorolignin derivatives are formed giving rise to the characteristic dark color [1]. Black liquor from kraft pulping process contains a blend of alkali lignins; its intense brown color along with its recalcitrance to biodegradation makes this residue an issue of environmental concern. Many pulp mills discharge into the environment their pulping effluents with insufficient or no treatment [2, 3] causing a dramatic increase in environmental pollution [4] particularly to aquatic ecosystems [5]. Acid precipitation of lignin is a commonly applied treatment to black liquor, after precipitation of more than 90% of lignin is removed from the solution as a solid material [6]. Nevertheless, the remaining soluble percentage is composed of oxidized and partially degraded lignin (predominantly composed of oligomeric lignin compounds) whose dark brown color is not only aesthetically unacceptable, but could also inhibit the process of photosynthesis in natural aquatic environments due to the barrier effect to sunlight. In addition, the precipitated lignin generates large volumes of sludge, which requires further treatment and disposal.

Lignin is difficult to biodegrade; white-rot fungi are the unique organisms able to degrade lignin efficiently to a complete mineralization. Under favorable environmental conditions, wood attacked by these fungi could lose up to 90% lignin content [7]. The high capability of white-rot fungi to degrade lignin is based principally on the activity of ligninolytic enzymes. These fungi secrete a system of extracellular nonspecific enzymes characterized by possessing a high redox potential. Ligninolytic system is made up of at least three enzyme activities: lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. The same unique nonspecific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants. In fact, these enzymes have demonstrated to be capable to degrade a vast number of different environmental contaminants, including: dyes, polychlorinated biphenyls, and pesticides [8], making ligninolytic enzymes potential efficient tools for biotechnological processes of bioremediation.

The objective of this work was to evaluate the capacity of selected strains of white-rot fungi to decolorize black liquor in plates, submerged fermentation and solid-state fermentation. White-rot fungi strains were cultivated under different culture conditions along with different black liquor concentrations. Growth, black liquor decolorization and ligninolytic enzyme production were studied.

Materials and Methods

Fungal Strains

Coriolus antarcticus BAFC 266, *Phanerochaete sordida* BAFC 2122, *Pycnoporus sanguineus* BAFC 2126, *Steccherinum* sp. BAFC 1171, and *Trametes elegans* 2127. All

of the strains are deposited in the culture collection of Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. The fungal cultures were maintained on agar (2%) and malt extract (13%; MEA) and glucose (1%) slants at 4 °C with periodic transfer.

Black Liquor Preparation

Solid content of kraft black liquor was determined after drying 1 mL of black liquor at 80 °C to constant weight. Black liquor was dialyzed against distilled water at 1:1,000 ratio (*v*/*v*) by using a latex membrane cut off of 2.5 kDa. Distilled water was replaced each 24 h.

Decolorization on Agar Plates

As a preliminary step, agar plates supplemented with 2%, 4%, and 10% *v*/*v* black liquor were inoculated with the five strains. Two culture media were assayed: MG and AG. AG medium contained the following per liter: glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.4 mg; $MnCl_2 \cdot 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; and asparagine monohydrate, 4 g. MG medium contained the following per liter: glucose 10 g and malt extract 13 g. Both media were adjusted to pH 6.5 with HCl 1 N. Inoculum consisted of one 25-mm^2 agar plug of a 10-day-old culture grown on MG. A control plate with no black liquor added was also inoculated with each strain. Uninoculated plates served as controls for abiotic decolorization. Plates were incubated at 28 °C for 45 days. Growth was followed by measuring radial extension of mycelium. A decolorized halo appeared when the fungus degraded the black liquor. Daily measurements of the colonies and the decolorized halo (if any) were performed for each strain. Measurements ended when mycelia reached the border of the plate. The measurements (cm day^{-1}) reported are the mean of triplicate assays with a standard deviation of less than 5%.

Decolorization in Liquid Media

Decolorization in liquid cultures was performed in Erlenmeyer flasks using GA and ME broth supplemented with different black liquor concentrations chosen among 2%, 4%, and 10% (*v*/*v*). These treatments were also applied to cultures grown for 15 days. The cultures were filtrated, and the supernatants were then centrifuged at 3,000×*g* for 10 min; the aqueous phases were collected, and decolorization was estimated.

Decolorization in Solid-State Fermentation

Decolorization in solid-state fermentation (SSF) was tested in 125 mL Erlenmeyer flasks by using two solid media: vermiculite (1 g) plus GA or ME media as liquid phase. Water content of all of these solid media was 85% (*v*/*w*). Three aqueous black liquor concentrations were assayed 10%, 4%, and 2% (*v*/*v*). Media were then autoclaved for 20 min at 121 °C. These Erlenmeyer flasks were inoculated with one 25-mm agar plug of a 10-day-old culture of each fungal strain grown on MEA. A control flask with no black liquor added was also inoculated with each strain. Uninoculated flasks served as controls for possible abiotic color transformation and as blanks of color where no decolorization occurred. Each fungus was tested in three independent experiments. Erlenmeyer flasks were incubated at 28 °C, experiments lasted 2 months. After 15, 30, and 60 days, three cultivation flasks of each treatment along with three untreated flasks were harvested. Crude extracts were obtained by

adding 5 mL (g humid medium)$^{-1}$ of distilled water to the contents of each flasks, stirring for 20 min, followed by filtration and centrifugation. The crude extracts were stored at −20 °C until needed. All analytical determinations were made by using these crude extracts.

Estimation of Black Liquor Decolorization

Liquid crude extracts were scanned using a UV–vis Spectrophotometer (PerkinElmer WinLab-5.1). Areas under spectral curves (AUC) were obtained using the program ImageJ version 1.42q. Decolorization was expressed as percentage reduction in absorbance area according to the equation:

$$\text{Decolorization}(\%) = \text{AUC}_x \times (\text{AUC}_{\text{control}})^{-1} \times 100 \quad (1)$$

Where AUC_x = area under spectral curve of crude extract from fungal treated black liquor; $\text{AUC}_{\text{control}}$ = area under spectrum curve of crude extract from uninoculated flasks. Visible range scanned was 400 to 680 nm. Solutions with different concentrations of black liquor were scanned to determine differences in the spectral curves. The area under the curves was plotted as function of black liquor concentration and the calibration curve was estimated by linear regression. For different fungal treatments, a straight baseline was drawn from the lowest point between 400 and 680 nm.

Enzyme Assays

All enzyme activities were assayed at 30 °C. Laccase activity was determined by oxidation of DMP (2,6 dimetoxyphenol) (ε_{469}=27 mM^{-1} cm^{-1}). The reaction mixture contained 0.1 M sodium acetate buffer, pH 3.6 and 5 mM DMP [9]. MnP activity was determined by oxidation of phenol red (0.01%). The reaction product was measured at 610 nm (ε_{610}= 22 mM^{-1} cm^{-1}). The reaction mixture contained 0.05 M succinate buffer pH 4.8 and 0.1 mM $MnSO_4$; reaction was initiated by the addition of 0.1 mM H_2O_2 [10].

Results

Decolorization on Agar Plates

Original black liquor was dialyzed through latex membrane (2.5 kDa cut off) against distilled water to remove salts. The solid contents of the dialyzed and the original black liquor were 140 and 270 g L^{-1}, respectively. Plates at 0.5% *v*/*v* of original (not dialyzed) black liquor concentration were inoculated with the fungal strains, but none of the fungi grew after 30 days of incubation period. Then, salts of the original black liquor were removed through dialysis; as a consequence, the pH was reduced to 8. Consequently, the subsequent experiments were performed by using dialyzed black liquor. The growth of different fungi on different culture media, a defined medium AG and a natural medium MG, at three concentrations of dialyzed black liquor (2%, 4%, and 10% [*v*/*v*]) was studied. These concentrations of black liquor were equivalent to 0.28%, 0.56%, and 1.4% (*w*/*v*), respectively. Halos of mycelial growth and black liquor decolorization by fungi growing on agar plates were daily measured.

The studied fungal strains were able to grow in all the assayed conditions even at the highest black liquor concentration tested, although not all of them yielded appreciable

decolorization (Table 1). Cultures on MG showed that *P. sordida*, *P. sanguineus*, and *T. elegans* of the five strains were able to decolorize the black liquor at the two concentrations assayed. While none of the cultures on AG decolorized the black liquor at any concentration assayed. Based on Tukey test, the fungi could be grouped into four categories regarding growth velocity (Table 1). *Steccherinum* sp. exhibited the more rapid growth among strains assayed, but no appreciable decolorization was observed. On the other hand, *P. sordida* and *P. sanguineus* were able to decolorize the black liquor forming an evident halo on plates, but their growth velocities were slightly slower.

Decolorization in Liquid Cultures

None of the fungi were able to decolorize the black liquor in liquid cultures irrespectively whether it was added to fungal cultures that previously were precultured for 15 days (data are not shown). Two different culture media (GA and ME) along with three concentrations of black liquor (2%, 4%, and 10% [*v*/*v*]) were tested, but decolorization was undetectable. The recovery values up to 100% demonstrated that neither a decolorization occurred nor the fungal mycelia adsorbed the black liquor.

Table 1 Growth and decolorization on plates by the five selected fungal strains using two concentrations of black liquor in the culture medium, 4% and 2% (*v*/*v*)

Fungal strain	Medium	Dilution (% *v*/*v*)	Growth rate[a] (cm day^{-1})	Decolorization rate[1] (cm day^{-1})
BAFC 266	AG	4	0.84b	ND
		2	1.20c	ND
	MG	4	0.72b	ND
		2	1.04c	ND
BAFC 1171	AG	4	1.46d	ND
		2	1.50d	ND
	MG	4	1.56d	ND
		2	1.56d	ND
BAFC 2122	AG	4	1.12c	ND
		2	1.33c	ND
	MG	4	1.06c	1.25c (9)
		2	1.10c	0.61b (7)
BAFC 2126	AG	4	0.65a	ND
		2	0.96c	ND
	MG	4	0.82b	0.83b (11)
		2	1.13c	1.25c (7)
BAFC 2127	AG	4	1.27c	ND
		2	1.31c	ND
	MG	4	1.00b,c	0.53a (11)
		2	1.10c	0.45a (9)

Values are means of three independent experiments (SD<5%). The first day at which a decolorization halo was observed and measured is indicated between brackets

BAFC 266 C. antarcticus, *BAFC 2122 P. sordida*, *BAFC 2126 P. sanguineus*, *BAFC 1171 Steccherinum* sp., *BAFC 2127 T. elegans*, *ND* no decolorization was observed, *AG* asparagine–glucose medium, *MG* malt extract–glucose medium

[a] Means with the same letter are not significantly different ($p<0.05$)

Decolorization on SSF

The ability of the fungi to decolorize black liquor in SSF, by using vermiculite as solid support, was also examined. The fungal growth in SSF was daily examined by naked eye; the strains exhibited similar velocity in mycelial colonization of substrate. The mycelium of *P. sanguineus* showed at the thirtieth day of growth an intense orange color due to cinnabarin production. Compact and white mycelia produced by *C. antarcticus* covered entirely the substrate. *P. sordida*, *T. elegans*, and *Steccherinum* sp. showed typical mycelia of lax hyphae. Cultures on solid media exhibited a variable extent of black liquor decolorization depending on the culture day, fungal strain, culture media, and black liquor concentration. Decolorization percentages in crude extracts obtained from the SSF cultures at three time intervals of 15, 30, and 60 days were estimated. For the purpose of these analyses, the spectra of serial dilutions of black liquor were determined spectrophotometrically in the range 400–680 nm. The serial dilutions of black liquor in distilled water included 0.5%, 2%, and 4% (*v*/*v*). A first-order fit of measured areas versus concentration of black liquor was performed (Fig. 1). Data were fitted by linear regression (R^2=0.99). Spectra from black liquor after fungal decolorization compared to spectra from dilutions of original black liquor showed to be almost parallel in the range 430–520 nm; while in the range 400–430 nm, the decolorized black liquor showed a more pronounced increase as wavelength decreases (Fig. 2). Differences in the area values of different black liquor concentration at the spectral range 400–520 nm comparing to those at 400–680 were similar, thus the range 400–520 nm was adopted as the limits for calculating areas under the spectral curves. As the black liquor is a mixture of various different molecules, the decolorization could be better estimated when the more representative range of wavelengths is measured. Since both spectral areas from serial dilutions showed to be a linear function of concentrations and spectral curves of different concentrations of black liquor were similar to those of decolorized black liquor, the decolorization was estimated from the area under spectral curves.

Decolorization of black liquor in SSF cultures demonstrated that this recalcitrant pollutant could be effectively decolorized at different extent by the strains assayed (Table 2). All of the strains assayed showed that they are able to significantly decolorize the black liquor after 15 days of cultivation. *P. sordida* and *T. elegans* exhibited the highest decolorization values in MG and AG, respectively. *Steccherinum* sp. was able to decolorize up to 92% (520 mg mL^{-1}) after 60 days of cultivation, the highest decolorization percentage reached by the five strains assayed.

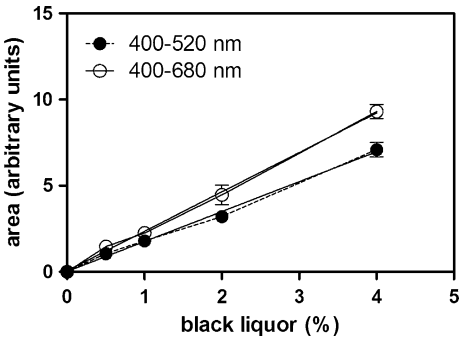

Fig. 1 Linear regression of measured areas versus concentration of black liquor

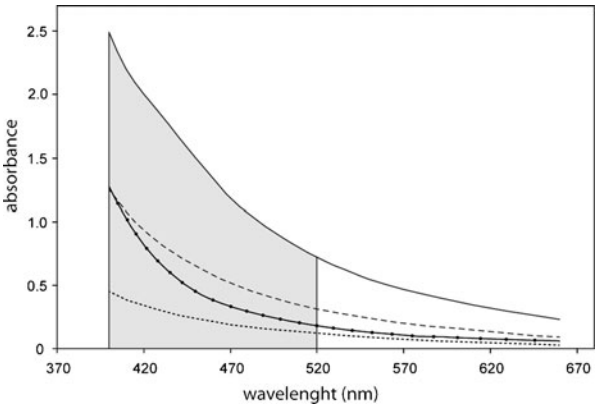

Fig. 2 Area under spectral curves (AUC) of 4% (*solid line*), 2% (*dashed line*), and 0.5% (*dotted line*) black liquor concentration. As an example, the spectral curve of fungal decolorized black liquor (strain 2122, after 60 days in MG with 4% black liquor) was also plotted (*circles*). *Gray area* indicates the selected zone as the representative area to calculate decolorization by the fungi

Ligninolytic Activities

During the different experiments, laccase activity was detected in all the strains, but neither LiP nor manganese peroxidase activities were detected. Similar values and low production of laccase were observed for all the fungi (Table 3). The highest values were observed after 60 cultivation days in *C. antarcticus* and *P. sanguineus* in AG medium with 2% and 4% of black liquor added, respectively.

Discussion

The most significant source of pollution among different steps of paper making is pulping, which generates black liquor, a high-strength effluent. This effluent contains small suspended wood fragments, solubilized wood components (predominantly lignin), and salts. The composition of kraft black liquor depends mainly upon the type of process and the type of wood chips utilized. Pokhrel and Viraraghavan summarized the general characteristics of wastewater from paper making [4]. Since the high salt concentration in the kraft black liquor inhibited the growth of all fungi assayed, it should obligate removing

Table 2 Decolorization percentages at 15 and 60 days by the five selected strains during the decolorization of two concentrations (2% and 4% [*v*/*v*]) of black liquor under solid-state fermentation with MG or AG as nutritive medium

Strain	AG 4%		AG 2%		MG 4%		MG 2%	
	15 d	60 d	15 d	60 d	15 d	60 d	15 d	60 d
BAFC 266	37.27	44.00	ND	ND	32.00	88.52	35.36	76.53
BAFC 1171	15.82	42.69	17.38	39.09	29.36	91.86	30.95	87.64
BAFC 2122	23.80	33.69	56.13	52.29	55.59	60.08	60.43	85.24
BAFC 2126	21.11	19.77	20.24	18.68	6.14	82.03	34.31	72.91
BAFC 2127	39.25	31.96	53.18	56.56	ND	ND	17.59	70.06

The values are the mean of three independent replications, SD<5%. Since the decolorization data at 30 days were similar but lower than those observed at 60 days, these data are not shown

ND not determined

Table 3 Laccase production (U g^{-1} vermiculite) at 15 and 60 days by the five selected strains during decolorization of two concentrations (2% and 4% [*v*/*v*]) of black liquor under solid-state fermentation with MG or AG as nutritive medium

Strain	AG 4%		AG 2%		MG 4%		MG 2%	
	15 days	60 days	15 days	60 days	15 days	60 days	15 days	60 days
BAFC 266	1.29	3.49	0.96	5.41	1.18	2.14	0.95	2.55
BAFC 1171	0.5	0.80	0.33	0.52	1.42	2.22	0.56	3.22
BAFC 2122	0.45	0.77	0.32	0.38	1.55	1.94	0.72	2.22
BAFC 2126	1.26	3.99	0.55	1.77	0.86	2.38	0.75	2.41
BAFC 2127	ND	0.64	ND	0	0.92	2.49	0.75	1.8

The values are the mean of three independent replications, SD<5%

salts previously to attempt its biodegradation in nature. For this purpose, the use of membrane separation process in the treatment of wastewater and groundwater containing toxic metal ions proved to be a suitable technique. Fortunately, these dialyzing processes are being investigated and improved for similar purposes [11]. Decolorization experiments on plates exhibited a darker halo, it appeared before decolorization occurred. This browning effect was also observed when lignosulfonates were incubated with laccase; this enzyme was able to carry out a process of polymerization and depolymerization with a concomitant darkening increase of the solution [12]. Similar polymerization activity undertaken by laccase was also observed in ultra-filtered lignin from black liquor [13]. These two sequential steps, darkening followed by decolorization, suggested that at least two reactions may occur prior to the observed decolorization.

The almost complete removal of the major visible light absorbance areas observed after fungal treatment of black liquor suggested a degradation of all the chromogenic molecules contained in the black liquor. Although fungi may be able to decolorize dyes, the mineralization of them is uncertain; since decolorization implies, at least, degradation or destruction of the chromogenic molecule, the mineralization implies degradation to carbon dioxide and water [14, 15]. A serious problem, when the decolorization of mixtures of chromogenic molecules is estimated at a unique wavelength, is the representativeness of the absorbance measured to quantitatively estimate the decolorization. In this study, in agreement with other reports [14, 16, 17], the decolorization percentages were estimated from the area of spectral curves at visible range; these measurements should be more representative since decolorization of the total molecules was considered.

Apart from decolorization by white-rot fungi, adsorption of lignin onto lignocellulosic materials was a different solution proposed to bioremediate contamination with black liquor [18], but a current limitation of such process is the final disposal of the solid utilized [19]. Anaerobic process was considered another suitable treatment, but many authors asserted that treatments with anaerobic systems could be more efficient to represent a promising alternative [4, 20, 21]. Based on decolorization percentages achieved (up to 92%) by the fungi tested, decolorization of effluents by white-rot fungi could be an attractive alternative or a complement of the existing technologies such as adsorption, precipitation, and membrane filtration. Although white-rot fungi have the ability to degrade lignin and chlorinated lignin derivatives effectively [22], lignin cannot serve as the sole carbon source for these fungi because the degradation of lignin is apparently a very energy intensive process [23]. Decolorization of effluent from a bleach plant by cultures of white-rot fungi

has been demonstrated in numerous reports. Among the fungi used, *Trametes versicolor* has been the more efficient, achieving 92% of color reduction [1]. In terms of the amount of decolorized lignin, the values achieved by the five strains tested were the highest obtained to date (up to 5.2 g L^{-1} observed in cultures of *Steccherinum* sp. and *C. antarcticus*).

Studies on the optimization of growth conditions and ligninolytic enzymes production using white-rot fungi aimed to improve processes of black liquor decolorization are being reported [24, 25]. Regarding the involvement of ligninolytic enzymes in decolorization, intriguing results have been reported. Molasses decolorization and MnP production by *Flavodon flavus* were inversely correlated, suggesting no role for MnP in this process [26]. Font et al. observed that LiP and MnP were not produced by *T. versicolor* in cultures containing black liquor, but this fungus is able to produce both enzymes, and laccase was proposed as a responsible enzyme of black liquor detoxification [27]. Similar to these previous reports, none of the fungi tested produced peroxidases and the only ligninolytic enzyme detected was laccase. As it was demonstrated in many reports, redox mediators expand the range of laccase substrates; in the presence of these compounds, the enzyme is able to oxidize non-phenolic aromatic structures which represent more than 80% of lignin polymer. Thus, apparent discrepancies observed after comparing laccase activities and decolorization percentages in media AG and MG (highest laccase activities were observed in strains 266 and 2126 but decolorization percentage was low) could be explained not only in terms of the titers of laccase activity, but also the presence of naturally occurring mediators in the malt extract or probably those produced by the fungus.

Since different pulping conditions render the composition and structure of black liquor compounds undefined, various parameters (e.g., production of ligninolytic enzymes) and cultivation strategies need to be considered in an effort to develop a successful treatment process.

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