Process Safety and Environmental Protection 168 (2022) 67–76

Contents lists available at ScienceDirect

Process Safety and Environmental Protection

journal homepage: www.journals.elsevier.com/process-safety-and-environmental-protection

Treatment of kraft black liquor using basidiomycete and ascomycete fungi

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ARTICLE INFO

Keywords:
Kraft black liquor
Phanerochaete chrysosporium
Aspergillus uvarum
Biological treatment
Phenolics removal
Fungal enzymes

ABSTRACT

The Kraft Black Liquor effluent generated by the pulp and paper industry is a highly alkaline solution with high chemical oxygen demand (COD), phenolic content, toxicity, and low biodegradability. Currently, it is usually concentrated and used as combustible in cogeneration systems. However, the profitability of this management depends on the unstable energy prices. As a possible alternative, in this work, different fungi have been used to treat this polluting wastewater. Tests were carried out at 25 ºC and 150 rpm for 10 days in a batch reactor. Two fungi capable of releasing suitable enzymes have been tested, i.e., *Aspergillus uvarum* and *Phanerochaete chrysosporium*. The effluent was treated with and without solids and with and without pH control. In all cases, the evolution of COD, biological oxygen demand (BOD_5), colour index, and the concentration of reducing sugars and phenolic compounds were analysed. Besides, the enzymatic activities manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac) were measured. Results showed that the presence or absence of solid did not affect the biodegradation process, achieving similar efficiencies. Bioremediation with *P. chrysosporium* allowed to obtain removals of COD, colour and phenolic compounds of 65 %, 37 % and 56 %, respectively, while *A. uvarum* achieved 61 %, 81 % and 67 %, for the best conditions tested. These results give good perspectives for application of both fungi for problematic industrial wastewaters, such as black liquor. It is especially interesting the good results obtained with *A. uvarum*, which has not been previously tested for the treatment of effluents from the paper industry.

1. Introduction

The pulp and paper production entails huge amounts of water consumption, which depends on the characteristics of the raw material and the type of produced paper. Consequently, huge amounts of complex, toxic and low biodegradable wastewater, that needs to be treated, are produced. Among these wastewaters, the Kraft Black Liquor (KBL) effluent, generated during the kraft pulping process, is considered the most problematic waste stream (Hubbe et al., 2016; Toczyłowska-Mamińska, 2017). During the kraft pulping process, the wood chips are digested at high temperature and pressure in a solution of sodium hydroxide and sodium sulphide called white liquor. This solution dissociates the lignin and cellulose fibres, giving a solid pulp and a liquid residue as final products. The solid pulp obtained is washed and continues to the manufacture process, while the liquid phase is mixed with the liquid residue from the pulp washing process to form the KBL (Pokhrel and Viraraghavan, 2004). This effluent is a dark colour and highly alkaline solution with high chemical oxygen demand and toxicity, and low biodegradability (Pola et al., 2021). It is mainly composed by lignin, chlorophenols, tannins, fatty acids, and soluble sodium salts, among others (Nikolskaya et al., 2019). Hence, this effluent must be adequately treated before being discharged to avoid adverse environmental damages on natural flora, fauna as well as aquatic bodies (Adhikari and Bhattacharyya, 2015).

The KBL is usually subjected to chemical processes so that inorganic compounds, such as Na_2S and Na_2CO_3, are recovered, and the lignin is used as energy source. During these treatments, KBL is concentrated by evaporation to achieve at least a solid concentration of 65 % and subsequently it is used as fuel in the facility itself, obtaining energy and ash rich in sodium salts (Kamali and Khodaparast, 2015; Mateos-Espejel et al., 2011; Pola et al., 2022). Although this is the most widely employed method for KBL management, entails certain operational and

Abbreviations: KBL, Kraft black liquor; COD, Chemical oxygen demand; BOD, Biological oxygen demand; MnP, Manganese peroxidase; LiP, Lignin peroxidase; Lac, Laccase; PHAs, Polycyclic aromatic hydrocarbons; MEA, Malt extract agar; SD, Standard deviation; DNS, dinitrosalicylic acid; CN, colour number; SAC, Spectral absorbance coefficient; ABS, absorbance.

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https://doi.org/10.1016/j.psep.2022.09.065
Received 21 April 2022; Received in revised form 26 September 2022; Accepted 26 September 2022
Available online 28 September 2022

environmental issues, i.e., KBL viscosity that favours the plugging of pipelines, the formation of deposits and fumes of inorganics salts in evaporators and furnaces and the emission of odours and hazardous gases (Al-Kaabi et al., 2020; Kinnarinen et al., 2016; Pola et al., 2022), together with the energy demanded for the concentration step. The pulp and paper production are considered an energy-intensive industry because of its high dependence on electricity consumption. Both, electric and thermal energies are produced in cogeneration systems that are fed with natural gas and waste biomass (including KBL), even selling the surplus of the electric energy production. In addition, it is necessary to keep in mind that the profitability of this solution for KBL is based on a weak equilibrium strongly dependent on the international energy market that determines the national prices for gas and electricity. As a consequence of the current political situation, for several months, energy prices in the European Union (EU) have been at sustained and unprecedentedly high levels. This situation has raised the alarms and some European pulp and paper factories have already threatened with ceasing of activity. Unquestionably, the economic sustainability of these industries will strongly depend on the buy price of fuels and the sell price of the surplus electricity.

All above commented makes interesting the investigation of other possible alternatives for the management of KBL based on their treatment. Other physical-chemical techniques such as wet air oxidation, ozonation, electrocoagulation, or membrane technologies have been studied in order to reduce its colour or its concentration of recalcitrant compounds (Bijan and Mohseni, 2004; Coimbra et al., 2021; Efgan Kibar et al., 2019; Ribeiro et al., 2020) or to recovery some valuable compounds such as lignin (Morya et al., 2022; Pola et al., 2022), organic acids (Niemi et al., 2011; Núñez et al., 2022), aliphatic acids (Kumar and Alén, 2014), polysaccharides (Lisboa et al., 2005), or different aromatic compounds (Heeres et al., 2018). Although the application of these methods has been shown to be effective, many disadvantages are related to their use, mainly associated to the operating cost. In addition, undesirable by-products such as aliphatic acids or phenolic compounds could be generated during some of these treatments (Hassan et al., 2018).

It is frequent that biological methods can suitably overcome some of the drawbacks of physic-chemical techniques since they allow the transformation of the toxic chemicals to less harmful forms in an economical and eco-friendly way (Madan et al., 2018). Both aerobic and anaerobic treatment techniques have been described previously for the treatment of paper industry wastewater achieving a partial removal of organic matter and phenolics compounds (Abhishek et al., 2017; Brink et al., 2017; Vashi et al., 2018). However, the composition of KBL makes impossible to treat it by a conventional biological process. The removal of KBL recalcitrant pollutants in not an easy task for bacteria, mainly because of the presence of complex structures such as lignin or chlorinated lignin compounds (Hassan et al., 2018). In this sense, some researchers have focused on looking for suitable microorganisms that allow the degradation of these complex compounds, improving the effectiveness of the biological treatment process (Brown et al., 2021; Hooda et al., 2015).

Fungal treatment has been considered a promising alternative for the treatment of complex industrial effluents due to the capability of some fungi to synthetise extracellular enzymes, including lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac) (Collado et al., 2019; Díaz et al., 2022b). Unlike bacteria, fungi have strong adaptability and can resist adverse conditions of temperature and pH, as well as limited nutrient availability (Espinosa-Ortiz et al., 2016). The release of these nonspecific enzymes allows them to degrade a wide variety of recalcitrant contaminants such as pesticides, fuels, polycyclic aromatic hydrocarbons (PAHs), and synthetic dyes (Rodríguez-Couto, 2017).

The use of fungi to treat industrial wastewaters in order to degrade complex compounds such as humic acids, phenols, azo dyes or pharmaceutical products have been deeply studied in the last decades (Asif et al., 2017; Collado et al., 2018, Ortiz-Monsalve et al., 2019). Specifically, some authors have tested the application of white-rot fungi and its enzymes for the treatment of KBL. Font et al. (2003), have reported reductions in COD and colour higher than 60 % after the treatment of black liquor by *Trametes versicolor* in the form of pellets in aerated reactors. Costa et al. (2017) have studied the capacity of *Phanerochaete chrysosporium* and *Bierkandera adusta* to degrade lignin, reaching removal values up to 74 % and 97 %, respectively, in synthetic pulp mill wastewater. Moreover, both fungi were able to achieve 100 % of delignification when real pulp mill wastewater was used. The fungi within the black *Aspergillus* group have been also described as good degraders for the wastewaters from the paper industry. Regarding phenolic compounds, an elimination around 60 % was accomplished using *Aspergillus niger* in batch treatment of real pulp mill effluent (Sharma and Gupta, 2012; Sharma and Rath, 2021). Thus, considering all the above reasons, fungi were selected to carry out the biological treatment of KBL in this study.

The aim of this study was to evaluate the capacity of the ascomycete fungus *Aspergillus uvarum* and the basidiomycete fungus *Phanerochaete chrysosporium* to remove phenolic compounds, reducing sugar, colour, recalcitrant organic matter and/or enhance the biodegradability of KBL. As far as we know, the application of *A. uvarum* to treat this industrial wastewater has not been previously reported. Its behaviour is compared with the use of *P.chrysosporium* that has previously given good results for the treatment of similar effluents. The influence on the treatment of pH and the possible effect of a filtering previous step were also evaluated.

2. Material and methods

2.1. Sample description

The kraft black liquor (KBL) corresponds with the waste stream obtained after the cooking step from *Eucalyptus* wood, which was provided by a paper mill located in Asturias (Spain). A detailed information about it physicochemical composition can be seen in Pola et al. (2021). The pH value of the raw KBL was 12.7. For the biological treatment, the raw KBL was diluted with distilled water in a ratio of 1:25 and the pH was adjusted to a value of 6.0 (KBL 1) or 4.0 (KBL 2), using NaOH 1 M (and HCl 1 M. The characterisation of the diluted and pH adjusted KBL used for the experiments is showed in Table 1.

The concentration of COD in the raw KBL liquor is very high (around 140 mg O_2/L). This fact hinders the growth of microorganisms used for subsequent biological treatment. To avoid inhibition of fungal growth, several plate growth tests were performed using KBL diluted with distilled water in ratios 1:10, 1:25 and 1:50 (data not shown). All agar plates were incubated at 25 °C for 20 days. After this time, on agar plates with a ratio 1:10 of KBL, fungi growth inhibition was observed, whereas no negative effects were observed for dilutions higher than 1:25. This is the reason for the dilution selected. Although it may seem that a significant amount of water is required to be added, this fact could be solved by mixing the KBL with other wastewater streams that need to be treated.

Table 1
Characteristics of KBL diluted 1:24 after pH adjusting.

Parameter	Value	
	KBL 1	KBL 2
pH	6,03 ± 0,01	4,03 ± 0,03
sCOD (mg O_2/L)	5792 ± 68	3590 ± 44
$sBOD_5$ (mg O_2/L)	443 ± 10	291 ± 13
Biodegradability (BI)	0,08 ± 0001	0,06 ± 0001
Soluble reducing sugars (mg/L)	393 ± 16	380 ± 13
Soluble phenolic compounds (mg/L)	629 ± 11	587 ± 8
Colour Number (CN)	3,4 ± 0,1	2,0 ± 0,1
Dry matter (%)	7 ± 0,1	9 ± 0,1

2.2. Microorganisms and culture conditions

Aspergillus uvarum MUM 08.01 and *Phanerochaete chrysosporium* MUM 95.01 were obtained from MUM culture collection (University of Minho, Braga, Portugal). The fungi were grown on 2 % of malt extract agar (MEA) at 26 ºC for 7 days.

To obtain the *A. uvarum* fungal pellets, a small portion of fungal biomass from the growing zone of petri dish was scraped with a smear loop and used to inoculate a 250 mL Erlenmeyer flask with 150 mL of Czapek-Yeast medium (1.3 g/L of K_2HPO_4, 5 g/L of yeast extract, 30 g/L of sucrose and 5 mL of a Czapeck concentrate), previously sterilised at 121 ºC for 20 min. The Czapeck concentrate consisted of 300 g/L of $NaNO_3$, 50 g/L of KCl, 50 g/L of $MgSO_4 0.7\ H_2O$, 1 g/L of $FeSO_4 0.7\ H_2O$, 1 g/L of $ZnSO_4 0.7\ H_2O$ and 0.5 g/L of $CuSO_4 0.5\ H_2O$. After 6 days, the pellets were obtained.

For the obtention of *P. chrysosporium* fungal pellets, the procedure described by Díaz et al. (2021b) was followed: five cylinders of 1 cm diameter from the growth zone of petri dishes were inoculated into a 500 mL Erlenmeyer flask containing 150 mL of ME broth, previously sterilised at 115 ºC for 10 min and pH adjusted between 4.5 and 5. Subsequently, flask was incubated at 26 ºC with constant orbital shaking at 135 rpm for 6 days. The fungal biomass obtained was separated from the liquid medium with a 1 mm mesh sieve, resuspended in 0.8 % (w/v) NaCl at a ratio of 1:3 (w/v) and mixed at 11,000 rpm for 5 min with a homogeniser (Heidolph Silent Crusher), thus obtaining the mycelial suspension. Pellets were obtained by inoculating 600 μL of this suspension into a 1 L Erlenmeyer flask with 250 mL of ME broth, which was incubated at 26 ºC with constant orbital shaking at 135 rpm for 6 days.

To determine the moisture of the pellets, the fungal biomass was oven-dried to constant weight at 105 ºC.

2.3. Batch experimental procedure

Six different batch experiments were carried out to treat non-sterile diluted KBL. The supplementation of the wastewater with an easily assimilable carbon source promotes fungal growth, enzymatic activity and enhance the degradation process of recalcitrant matter (Díaz et al., 2022a; Saetang and Babel, 2010). Based on a previous work that evaluated the optimal glucose addition for treating an industrial wastewater with fungi (Díaz et al., 2021b), the KBL was supplemented with 3 g/L. The operational conditions of each test are shown in Table 2.

As can be seen in Table 2, two experiments were carried out with *P. chrysosporium* MUM 95.01 using diluted KBL with solids and keeping the pH constant at a value of 4.0 (test P4) and 6.0 (test P6) by adding NaOH 1 M (Sigma-Aldrich, reagent grade, ≥98 %) or HCl 1 M (Sigma-Aldrich, ACS reagent, 37 %) during the treatments. These experiments were carried out in 1 L Erlenmeyer flasks containing 200 mL of sample inoculated with 3 g/L (dry matter) of fungus. Erlenmeyer flasks were incubated at 25 ºC for 10 days in an orbital shaking (New Brunswick Scientific, Excella E25) at 150 rpm.

Table 2
Experimental design for the biological treatments. The medium used was diluted KBL and the amount of inoculated fungus was 3 g/L (dry matter).

Test	Fungus	Initial pH	Glucose	pH control	Solids
P4	*P. chrysosporium*	4.0	3 g/L	Yes	Yes
C-P4	Non inoculated	4.0	3 g/L	Yes	Yes
P6	*P. chrysosporium*	6.0	3 g/L	Yes	Yes
C-P6	Non inoculated	6.0	3 g/L	Yes	Yes
U4	*A. uvarum*	4.0	3 g/L	Yes	Yes
C-U4	Non inoculated	4.0	3 g/L	Yes	Yes
U6	*A. uvarum*	6.0	3 g/L	Yes	Yes
C-U6	Non inoculated	6.0	3 g/L	Yes	Yes
US	*A. uvarum*	6.0	3 g/L	No	Yes
CS	Non inoculated	6.0	3 g/L	No	Yes
UL	*A. uvarum*	6.0	3 g/L	No	No
CL	Non inoculated	6.0	3 g/L	No	No

Two experiments were also carried out with *A. uvarum* MUM 08.01 in 500 mL Erlenmeyer flasks containing 100 mL of sample at pH 4.0 (test U4) and pH 6.0 (test U6). In both cases, the pH was controlled during the treatment. Again, the sample was inoculated with 3 g/L (dry matter) of fungus. The incubation conditions were the same as in the case of *P. chrysosporium*.

Finally, with the aim to evaluate the influence of pH control during the treatment, two additional experiments similar to U6 were carried out using diluted KBL adjusted initially to pH 6. Test US were performed with the raw diluted KBL, whereas test UL were carried out using diluted KBL previously centrifuged for 20 min at 9000 rpm and filtered by 0.45 μm filter (Millipore). In these cases, the pH was not controlled during the fungal treatment.

For all the conditions tested, control experiments without fungus addition were assayed concurrently, with the aim of evaluating the effect of fungus addition in relation with the activity of the endogenous microbiota. These control tests correspond to C-P4, C-P6, C-U4, C-U6, CL and CS.

In all cases, the experiments were carried out in duplicate. The data shown in Figs. 1, 2 and 3, are the average of the experimental data. In each particular case, standard deviation (SD) with respect to the mean value is shown as error bars in graphs. The percentage of SD with respect to average of the experimental data was less than 12 % in all cases. Periodically, samples were taken for the analysis of sCOD, total phenolic content, reducing sugars concentration, colour number, pH, $sBOD_5$ and enzymatic activities (Lac, MnP and LiP).

2.4. Analytical methods

Samples were centrifuged at 9000 rpm during 10 min (Kubota 6500 High Speed Refrigerated Centrifuge), filtered by 0.45 μm filter (Millipore) and stored at − 20 ºC until being analysed. All analytical measurements were done at least in triplicate.

Soluble biochemical oxygen demand ($sBOD_5$) was determined using a manometric respirometry measurement system (Lovibond® Water Testing BD 600). For soluble COD (sCOD) determination, the test kit Hach Lange LCK 514 was used, and samples was spectrophotometrically measured at 600 nm (HI 83224 Wastewater Treatment Spectrophotometer). The biodegradability index (BI) was calculated as the ratio of soluble $sBOD_5$ over soluble sCOD.

The concentration of reducing sugars was determined by the Miller method, using 3,5-dinitro-2-hydroxybenzoic acid (Sigma-Aldrich, reagent grade, ≥98%), according to the methodology described by Díaz et al. (2017). Glucose (Sigma-Aldrich, reagent grade, ≥99.5%) was used as standard. The absorbance was measured at 540 nm using a UV/vis spectrophotometer (Thermo Scientific, Heλios γ).

Total phenolic content was measured using by the Folin–Ciocalteu's phenol method following the procedures of Moussi et al. (2015). In this procedure, 400 μL of sample were mixed with 3 mL of Folin-Ciocalteu reagents (previously diluted 1:10 with distilled water). This mixture was maintained at 22 ºC for 5 min. After that, 3 mL of sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, ACS reagent, ≥99.7 %, 0.6 g/L) were added, and the sample was again incubated at 22 ºC for 90 min. After incubation, the absorbance was measured at 725 nm against a blank. Gallic acid (Sigma-Aldrich, 97.5–102.5 %) was used as standard.

The change in the colour of the KBL was indicated by the colour number (CN), which is defined according to Eq. (1), where spectral absorbance coefficients (SAC) are defined as the ratio of the values of the absorbances (Abs) measured at 436, 525 and 620 nm over the cell thickness (x) using a UV/VIS spectrophotometer (Thermo Scientific, Heλios γ) (Díaz et al., 2020).

$$CN = \frac{SAC_{436}^2 + SAC_{525}^2 + SAC_{620}^2}{SAC_{436} + SAC_{525} + SAC_{620}} \quad (1)$$

For the analysis of Lac, LiP and MnP enzymatic activities the

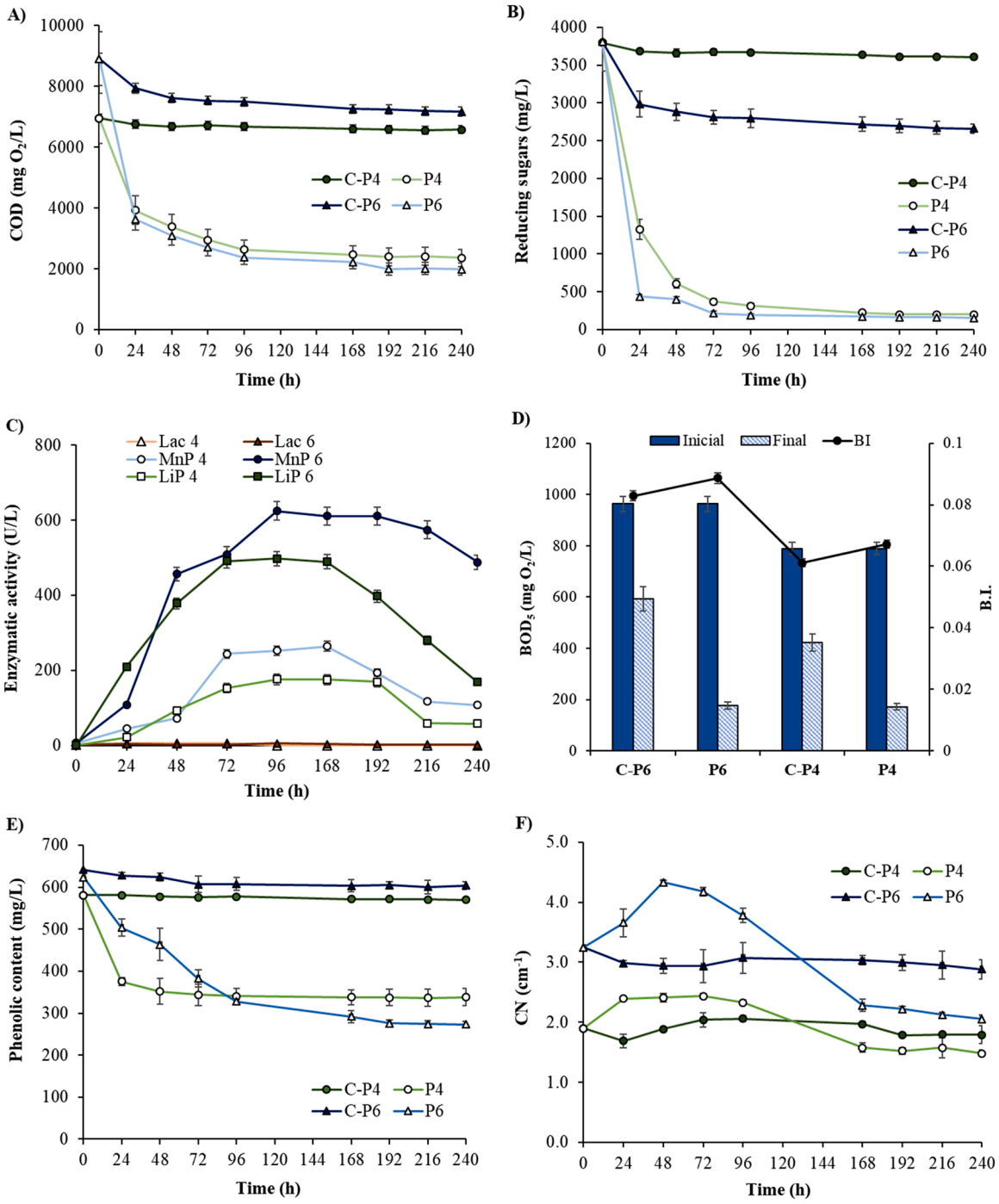

Fig. 1. Evolution of different parameters during the treatment of KBL with *P. chrysosporium* and pH control. (A) Soluble COD (B) Reducing sugars (C) Enzymatic activities (D) BOD_5 and biodegradability index (BI) (E) Phenolic compounds. (F) Colour number (CN). In A, B, E and F, empty markers show the inoculated tests, P6 (△) and P4 (○) and filled markers show the non-inoculated tests, C-P6 (▲) and C-P4 (●). In C, filled markers and empty markers show data of P6 and P4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. Standard deviations of each datum are shown.

methodology described by Lisboa et al. (2017) was followed. For Lac activity the reaction mixture was: 0.8 mL of 0.03 % ABTS (v/v), 0.1 mL sodium acetate buffer (0.1 M, pH 5.0) and 0.1 mL of sample. The oxidation of ABTS was measured at 420 nm with a molar extinction coefficient (ε) of 3.6×10^4 $M^{-1}cm^{-1}$. The LiP activity was assessed by mixing 1 mL of sodium tartrate buffer solution (125 mM, pH 3.0), 500 µL of veratryl alcohol (VWR, reagent grade, ≥98 %, 10 mM), 500 µL of hydrogen peroxide (Thermo Scientific, 2 mM) and 500 µL of sample. The production of veratraldehyde was measured at 310 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1}cm^{-1}$). The measurement of MnP activity was carried out using the Phenol Red' method by mixing 500 µL of sample, 100 µL of phenol red (Merck, 0.01% p/v), 100 µL of sodium lactate (250 mM), 200 µL of bovine albumin (Sigma-Aldrich, 0.5% w/v), 50 µL of sulphate manganese (Merck, 2 mM), 50 µL of hydrogen peroxide (2 mM) and 1.0 mL of sodium succinate buffer (20 mM, pH 4.0). All the reactions were performed at 30 °C for 5 min and were stopped by adding 40 µL of 2 N NaOH. One unit of enzyme (U) was defined as the release of 1 µmol product formed per min under the assay conditions. Finally, total solids

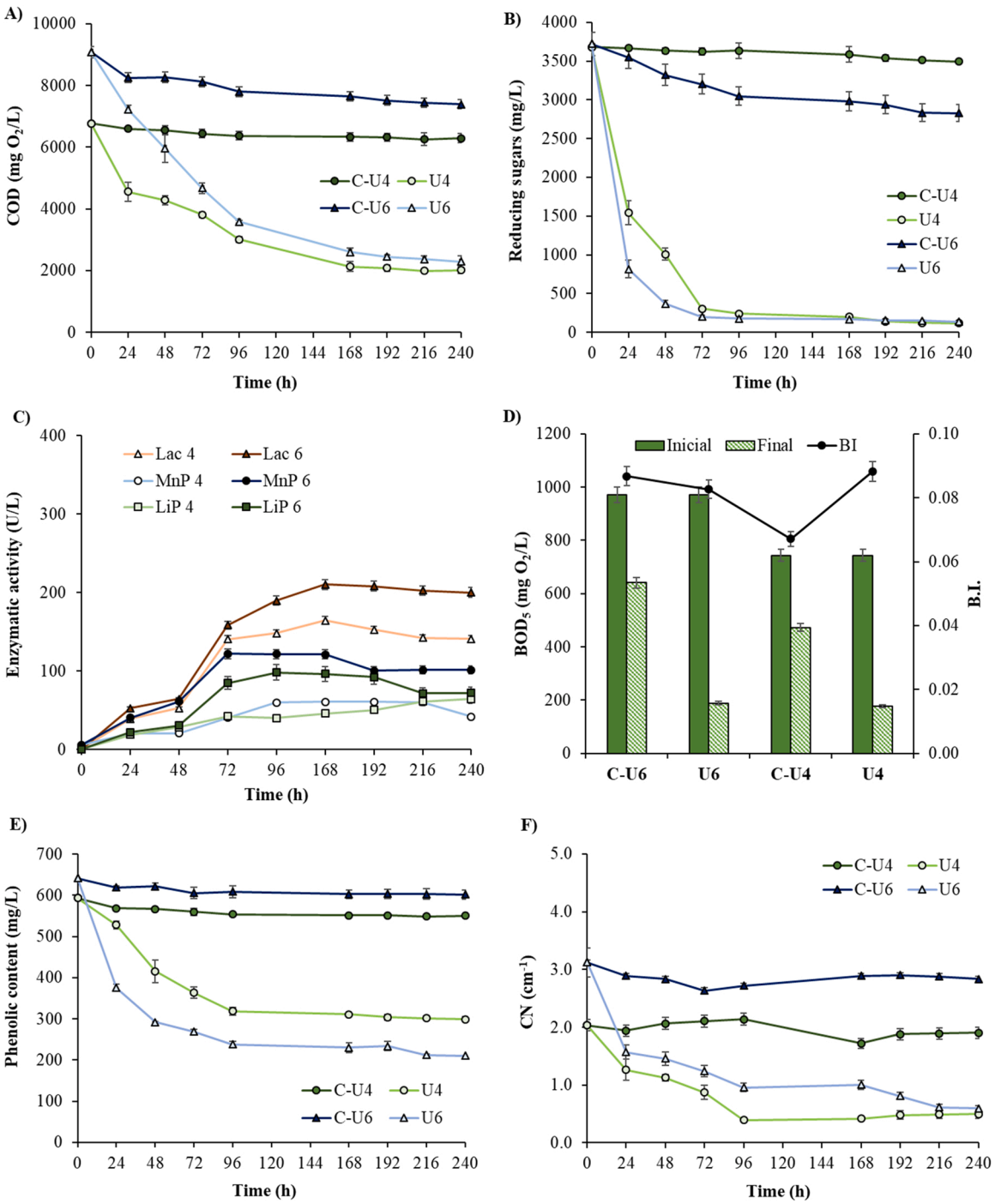

Fig. 2. Evolution of different parameters during the treatment of KBL with *A. uvarum* and pH control. (A) Soluble COD (B) Reducing sugars (C) Enzymatic activities (D) BOD_5 and biodegradability index (BI) (E) Phenolic compounds. (F) Colour number (CN). In A, B, E and F, empty markers show the inoculated tests, U6 (▲) and U4 (●) and filled markers show the non-inoculated tests, C-U6 (▲) and C-U4 (●). In C, filled markers and empty markers show data of U6 and U4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. Standard deviations of each datum are shown.

(TS) were analysed by oven-drying (105 °C) a determined volume of sample to constant weight and the value of pH was measured by means of a pH-metre (Jenway 3510).

3. Results and discussion

3.1. KBL biodegradation by P. chrysosporium with pH control

Diluted KBL was biotreated by *P. chrysosporium* at pH 4 (P4) and pH 6 (P6). As can be seen in Table 1, the characteristics of the raw material were different according to the initial pH, especially for sCOD, $sBOD_5$ and colour, surely due to the precipitation of lignin as a consequence of the pH adjustment.

Acid precipitation is the most commonly used technique to extract lignin from black liquor, with maximum efficiencies for pH values below 3. Strong acids, such as sulphuric acid or hydrochloric acid, can be used to precipitate Kraft lignin obtaining a treated effluent with lower COD and CN (Pola et al., 2019). In the tests carried out at pH 4, a partial lignin

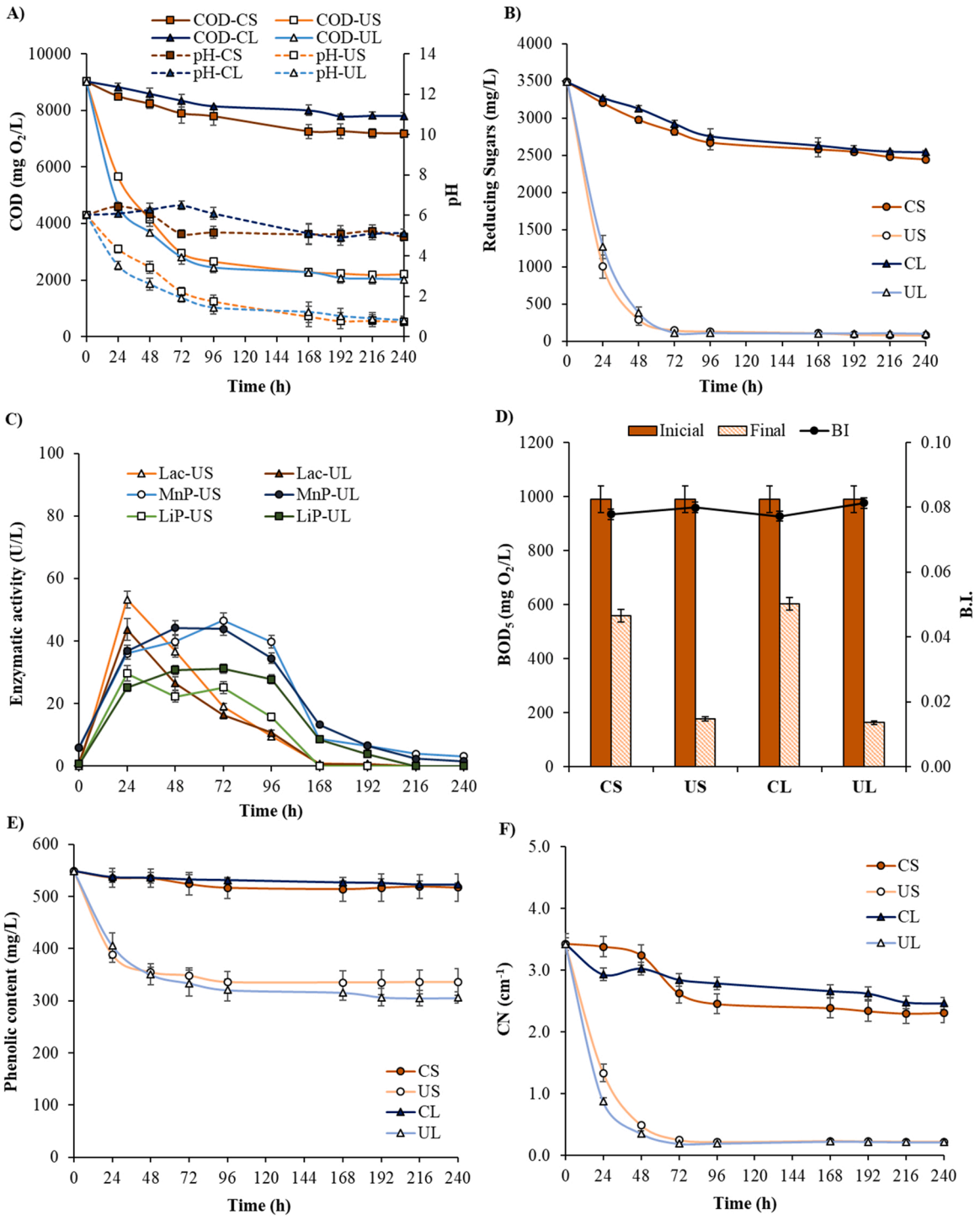

Fig. 3. Evolution of different parameters during the treatment of KBL with and without solids, and without pH control with *A. uvarum*. (A) Soluble COD (solid lines) and pH (dashed lines) (B) Reducing sugars (C) Enzymatic activities (D) BOD_5 and biodegradability index (BI) (E) Phenolic compounds. (F) Colour number (CN). In A, B, E and F, empty markers show the inoculated tests, UL (△) and US (○) and filled markers show the non-inoculated tests, CL (▲) and CS (●). In C, filled markers and empty markers show data of UL and US, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. Standard deviations of each datum are shown.

precipitation occurred, which was reflected in the increase of dry matter (from 7 % at pH 6.0–9 % at pH 4.0), and in the decrease of colour number (from 3.4 at pH 6.0–2.0 at pH 4.0). The evolution of soluble COD, soluble reducing sugars, soluble phenolic compounds, colour number (CN), soluble BOD_5 and enzymatic activities (LiP, MnP and Lac) for each experiment and control (CP4 and CP6), is shown in Fig. 1.

In relation to sCOD degradation (Fig. 1A), for the non-inoculated tests, the sCOD concentration of test C-P4 remained almost constant during the treatment, whereas test C-P6 showed a slight reduction, carried out by the endogenous microbiota from KBL. Despite of the high alkalinity of Kraft paper effluents, microorganisms capable to survive in these wastewaters, mainly within *Firmicutes* and *Proteobacteria* phyla have been identified and isolated in previous studies (Hooda et al., 2015). In both cases, the final sCOD concentration was higher than the

KBL before being supplemented with glucose. With respect to experiments inoculated with fungus (P4 and P6), a similar behaviour was observed, very different from controls. In both cases, a fast decrease of sCOD was observed during the first 24 h followed by a progressive degradation until the 4th day of treatment with a final concentration around 2000 mg/L. Afterwards, the sCOD concentration remained almost constant. However, as the initial sCOD was not the same in both cases, due to the lignin precipitation caused for the pH adjustment, the final COD removal achieved in relation with the initial sCOD concentration of the KBL before glucose addition, was much higher at pH 6 than at pH 4 (65 % and 35 %, respectively). Results here obtained were higher than the COD removals reported by Wu et al. (2005), who achieved COD removals less than 50 % after the treatment of diluted black liquor by *P. chrysosporium* and others white-rot fungi after 16 days of treatment at pH 6 and 28 ºC.

Regarding soluble reducing sugars evolution easily used by microorganisms (Fig. 1B), the addition of glucose increased the initial values from around 400 mg/L to 3800 mg/L. For the control test at pH 4, these values remained almost constant during the biological treatment, probably due to the protection of the acid pH, which avoid that glucose is consumed by environmental bacteria. At pH 6 a rapid decrease of reducing sugars was observed during the first 24 h, which corresponds with the reduction observed in sCOD. So, it can be concluded that the removal of sCOD observed in CP-6 was due to the consumption of the added glucose. It is important to keep in mind that the samples used in this work were not sterilised, so the endogenous microbiota from KBL could consume reducing carbohydrates. However, it is important to point out that even at pH 6 remained without being consumed around 2700 mg/L of reducing carbohydrates, probably due to the presence of inhibitory compounds, such as phenolics, which are in a concentration around 600 mg/L.

For the experiments inoculated with the fungus (P4 and P6), significant removals of reducing sugars were achieved compared with the initial one, reaching final values of 73 % and 64 % for P6 and P4, respectively. In both cases the elimination of reducing carbohydrates occurred mainly during the first 24 h (see Fig, 1B), with apparent consumption of 2.34 ± 0.02 mg/Lmin and 1.72 ± 0.07 mg /Lmin, at pH 6 and pH 4, respectively. It is necessary to take into account that the real consumption rate is higher because reducing sugars were producing at the same time as they are consumed thanks to the fungal enzymes that broke complex carbohydrates.

In this work, it was observed that regardless of the pH adjustment, the highest enzymatic activity was reached at around 96 h (see Fig. 1C), when most of the reducing carbohydrates had been consumed. According to results previously commented, the greatest activities were obtained at pH 6 with 625 ± 11 U/L and 497 ± 8 U/L for MnP and LiP, respectively. These enzymes are responsible for delignification, breaking down the lignin into by-products that the fungus can use as source of nutrients (Sharma and Rath, 2021). In addition, the removal of COD and colour has been mainly related with the release of these enzymes by white-rot fungi (Díaz et al., 2022a; Islam et al., 2019). Saetang and Babel (2010), who studied the treatment of landfill leachate by *Trametes versicolor*, reported higher COD (40 %) and colour (58 %) removals when the medium was supplemented with glucose. In that case, the enzyme activities measured were 384 U/L and 1241 U/L for LiP and MnP, respectively, whereas when no glucose was added, 193 U/L were obtained for LiP and 437 U/L for MnP. In this case, Lac activity was lower than 5 ± 0.6 U/L for both values.

Accordingly to the consumption of reducing sugars, a reduction of $sBOD_5$ concentration was obtained in all cases, reaching 172 ± 8 mg/L for test P4 and 196 ± 7 mg/L for P6 (Fig. 1D). However, due to COD removal, the use of *P. chrysosporium* gave final biodegradability index slightly higher than in the initial KBL, 0.07 and 0.09 for P4 and P6, respectively. Regarding control test, a certain reduction of $sBOD_5$ values was obtained because of glucose consumption by endogenous microbiota. However, these values were higher than the initial $sBOD_5$ of KBL, according to the fact that endogenous microorganisms could not degrade even the glucose added.

The evolution of phenolics concentration is shown in Fig. 1E. The removal of phenolic compounds is crucial because of its grave toxic effects on humans, animals and natural environment (Ariste et al., 2020). Again, the highest removals were reached for the experiment carried out at pH 6, where *P. chrysosporium* could degrade 56 % of phenolics after 8 days of treatment, with final concentration below 300 m/L. At pH 4, 44 % of phenolics were removed just in 3 days. For both controls tests the degradation was lower than 4 %. Although the dephenolization process is mainly related to the activity of Lac, this study probes that other ligninolytic enzymes, such LiP and MnP, also contributes to the degradation of these compounds. Results here obtained agree with other studies on the degradation of phenolics by white-rot fungi, i.e., Sampedro et al. (2007) reported removals around 43 % using free mycelia of *Phlebia* sp. to treat an olive mill effluent. Higher phenolic compounds degradations have been reported when the effluents to be treated were sterilised, i.e., García-García et al. (2000) reported a 92 % total phenol removal using *P. chrysosporium* to treat a sterilised OMW supplied with a nitrogen source.

The colour removal achieved by the treatment with *P. chrysosporium*, was lower than 30 % for both pH values. It should be noted that for both the pH values here assayed the colour of the sample was higher than the initial one during the first 96 h of treatment, which coincided with the drop of sCOD and the maximum enzymatic activity. Junnarkar et al. (2016) observed the production of a dark brown colour during the treatment of wheat straw by *P. chrysosporium*. This colour was related with secondary products formed during lignocellulosic material biodegradation. This fact might explain the behaviour here obtained, since *P. chrysosporium* could generate coloured by-products from lignin degradation. These CN increases have also been reported in previous works using this fungus to treat different wastewaters, i.e., olive mill effluents (Díaz et al., 2021a), anaerobic digestion liquors (Díaz et al., 2021b), or landfill leachates (Díaz et al., 2022a).

3.2. KBL biodegradation by A. uvarum with pH control

The industrial application of *A. uvarum* has hardly been studied. Therefore, its use for the treatment of KBL effluents has not been previously reported. This fungus was selected based on the good results reported by Salgado et al. (2016) for the treatment of olive mill and winery wastewaters, in terms of colour and phenolic compounds removal. *A. uvarum* belongs to the group of black Aspergilli and has the capacity to produce secalonic acid, asteric acid, geodyn, erdine and dihydrogeodyine (Perrone et al., 2008).

The evolution of sCOD for the KBL treatment by *A. uvarum* is shown in Fig. 2A.

In this case, the degradation of sCOD was progressive during the treatment with the inoculated fungus, whereas in the control tests sCOD remained almost constant at pH 4 and decreased slightly at pH 6. As occurred with *P. chrysosporium*, final sCOD was similar at both pH values. However, as the initial sCOD was higher at pH 6, the percentage of sCOD removal was higher at this pH. So, *A. uvarum* accomplished 61 % and 43 % of sCOD degradation at pH 6 and pH 4, respectively. Liu et al. (2011) reported similar results with a maximum COD removal of 60 % at pH 6.0 when an alkaline pulping effluent was treated by *Aspergillus niger*.

Regarding the evolution of reducing sugars concentration (Fig. 2B), again an abrupt decrease was observed during the first 72 h with degradation rates of 1.49 ± 0.11 mg/Lmin and 2.02 ± 0.08 mg/Lmin for U4 and U6, respectively. Compared to *P. chrysosporium*, the consumption of reducing sugars attained after 10 days was slightly higher, without significant differences between U4 and U6 (78% and 75%, respectively).

Compared to *P. chrysosporium*, the fungus *A. uvuarum* showed a lower capacity to synthesise LiP and MnP, with maximum activities achieved

at pH 6 of 98 ± 9 U/L and 122 ± 6 U/L, respectively (see Fig. 2C). On the contrary, enzymatic activity of Lac was much higher with *A. uvarum*, reaching values of 210 ± 7 U/L and 164 ± 12 U/L for U6 and U4, after 168 h of treatment. Previous works reported the ability of *A. uvarum* to synthesise other enzymes with proteolytic, lipolytic, and tannase activities (Salgado et al., 2014a,b), which together with the commented MnP, LiP and Lac, allowed the high efficacy of sCOD degradation.

Regarding BOD_5 concentration (Fig. 2D), the behaviour was similar to that obtained with *P. chrysosporium*. As soon as enzymes broke recalcitrant organic matter into compounds more biodegradable, they were consumed by the microorganisms. Final BI were also very low with values of 0.08 and 0.09 for U6 and U4, respectively.

The main different observed between the inoculation of *P. chrysosporium* or *A. uvarum* was observed with respect to the degradation of phenolic compounds, *A. uvarum* allowed to obtain higher removals for these kinds of pollutants, especially at pH 6 where 67 % of the phenolics were degraded (see Fig. 2E). This percentage of removal is higher to those reported previously, i.e., Salgado et al. (2015) who studied the use of this fungus to eliminate phenolic compounds from olive mill wastewater (OMW) in submerged fermentation, achieved removals of 28.32 % of phenolic compounds after 10 days of fermentation. Higher removals were obtained mixing OMW with vinasses, where around 43 % of phenolic compounds were reduced (Salgado et al., 2016).

At both pH values, the degradation process of phenolics was progressive until 96 h, and from this time on, concentration remained almost constant. The higher dephenolization observed is likely to be related with the higher Lac activity achieved with *A. uvarum*.

For both pH values, the use of *A. uvarum* gave better results in decolourization than those achieved with *P. chrysosporium* (Fig. 2F). The use of *A. uvarum*, gave a removal of 76 % and 81 % for U4 and U6 after 10 days, respectively. In both cases (U4 and U6), the reduction in the CN was in parallel with the reduction in phenolics concentration, with the only exception of the slight decolourization observed after 168 h of treatment at pH 6. This confirms the fact that the main responsible for the dark colour of KBL are phenolic compounds. Gulzar et al. (2017) reported decolourization efficacies of 78 % in synthetic textile effluent after 72 h of treatment by *Aspergillus niger*, whereas only 52 % was achieved for real wastewater, results quite lower than those achieved in this work.

3.3. Biodegradation of KBL without pH control in presence/absence of solids by A. uvarum

As results obtained in terms of sCOD degradation were similar for both microorganisms assayed, and phenolics degradation and consequently decolourization, was better with *A. uvarum*, this last fungus was selected for the next experiments. The qualities of the final effluents obtained at controlled pH 4 and 6 were quite similar, except for the concentration in phenolics that was lower at pH 6. Taking these results into account and in order to know if the control of pH during the treatment was necessary, the treatment of KBL with *A. uvarum* adjusting the initial pH at 6 and with free pH evolution during the treatment was carried out. Experiments were carried out with non-filtered KBL (with solids; US) and with filtered KBL (without solids; UL). This last experiment was carried out to test the possibility of treating the liquid phase after removing solids for being used to feed cogeneration systems.

The pH profiles are shown in Fig. 3A, as can be observed, a reduction of pH took place in all cases. The control tests (CS and CL) suffered a reduction of just one unit, reaching final values around 5.0 in both cases. On the contrary, in both inoculated test (US and UL), the pH value showed a fast drop during the first 96 h, reaching values below 2. After this time, pH continued to decrease progressively until reaching pH values around 1.0 after 10 days of treatment. The genus *Aspergillus* has been widely studied for its ability to produce organic acids such as citric, gluconic, or fumaric acid, using lignocellulosic materials as substrates (Dhakar et al., 2015). The organics acids may be responsible for the acidification observed in this work.

In Fig. 3B, the change of the concentration of soluble reducing carbohydrates is shown, with a behaviour very similar to that obtained with pH controlled at pH 6. It must be noted that, even for pH values below, sugars consumption was observed. Similar final BOD_5 values were observed for US and UL, with a BI of 0.08, as in U6 test (Fig. 3D).

With respect to the enzymatic activity, the values obtained without pH control was much lower than those obtained in the pH-controlled test. As can be seen in Fig. 3 C, the enzymatic activities were slightly higher for the US test, with maximum values of 47 ± 6 U/L for MnP and 31 ± 4 U/L LiP after 72 h, and 53 ± 5 U/L for Lac after 24 h. Then, the enzymatic activity drastically decreased, especially for Lac. This abrupt reduction in Lac activity happened when pH was less than 3.0. Dhakar et al. (2015) who studied the effect of temperature and pH on the production of ligninolytic enzymes (MnP, LiP and Lac) by *A. niger*, reported that the minimum production of Lac (0.8 U/L) corresponded to a pH 3.5, while at pH 7.5 the measured activity was 8.9 U/L. In addition, an increment in MnP and LiP production was described when pH increased considering pH 5.5 and pH 9.5 as optimum for MnP (1737 U/L) and LiP (215 U/L) production, respectively. In this work, the drop of pH suffered in UL and US, explains the low fungal enzymatic activities with respect to tests carried out at controlled.

Surprisingly, despite the pH drop and the low enzymatic activities, the sCOD degradation observed (Fig. 3A) in US and UL was very similar to that obtained with the controlled pH (U6). Removals over 60 % were achieved in presence or absence of solids, indicating that the enzymatic activities achieved in the first three days are enough to break most part of complex carbohydrates.

Some differences were observed for phenolics removal compared to tests carried out with pH control, where the enzymatic activity, especially Lac, was higher. In this case, efficiencies around 40 % were obtained for US and UL after 4 days of treatment (see Fig. 3E), whereas with pH controlled at pH 6 a percentage of 67 % was achieved. As stated before, the low pH significantly affected the enzymatic production by fungus and therefore the dephenolization process.

Finally, the changes in CN can be seen in Fig. 3F. In control samples (CS and CL) a colour removal close to 30 % took place, whereas the inoculation of the fungus allowed to obtain a colour removal of 94 %. In this case decolourization was due to phenolics degradation, but also by the acidification occurred during the treatment. In fact, acid precipitation is the most widely used technique to extract lignin. Normally strong acids such as sulfuric or hydrochloric are used to precipitate Kraft lignin obtaining a the treated effluent with lower COD and CN (Pola et al., 2019). In this work CN decreased until 72 h, indicating that a pH around 2 is enough to achieve almost a total decolourization. To sum up, similar results were obtained with and without solids and, with and without pH control (with the exception of phenolics concentration). So, a KBL treatment with *A. uvarum* without previous filtration and without pH control, which reduces operational costs, seems to be a good option to considerably reduce COD, phenolics and colour.

A summary of the final percentage removal obtained for COD, CN and phenolic compounds, according to the treatment performed, is shown in Table 3.

4. Conclusions

The white-rot fungus *P. chrysosporium* and the ascomycete fungus *A. uvarum* were tested for the treatment of non-sterilised KBL effluent as a possible alternative for its management in paper and pulp factories. Whereas very low biodegradations were observed in non-inoculated control test, both fungi were effective for the treatment of KBL, with higher efficiencies for the experiments carried out at pH 6, especially for phenolics degradations. In these controlled-pH tests, the fungus *P. chrysosporium* allowed to obtain removal efficiencies of 65 %, 50 % and 37 % for COD, phenolic compounds, and colour, respectively,

Table 3
Percentages of sCOD, colour, phenolic compounds and reducing sugars degradations at 10 days for inoculated tests (P4, P6, U4, U6, US and UL) and non-inoculated tests used as controls (C-P4, C-P6, C-U4, C-U6, CS and CL). Negative percentages of degradation indicate that the sample suffered an increase in the value of the parameter studied.

Test	sCOD	Colour	Phenolic compounds	Reducing sugars
P4	35 ± 0.3	22 ± 0.5	42 ± 0.3	64 ± 0.2
C-P4	-81 ± 0.2	5 ± 0.4	2 ± 0.1	-547 ± 1.3
P6	65 ± 0.3	37 ± 0.2	56 ± 0.7	73 ± 0.5
C-P6	-25 ± 0.1	11 ± 0.3	3 ± 0.2	-367 ± 1.1
U4	43 ± 0.6	76 ± 0.1	50 ± 0.9	78 ± 0.3
C-U4	-77 ± 0.4	6 ± 0.3	7 ± 0.3	-546 ± 1.2
U6	61 ± 0.3	81 ± 0.1	67 ± 0.8	75 ± 0.5
C-U6	-26 ± 0.1	9 ± 0.1	6 ± 0.2	-410 ± 1.1
US	62 ± 1.1	94 ± 0.7	39 ± 0.9	80 ± 0.3
CS	-24 ± 0.8	33 ± 0.5	6 ± 0.1	-519 ± 1.0
UL	65 ± 0.9	94 ± 0.6	44 ± 0.3	75 ± 0.3
CL	-35 ± 0.1	28 ± 0.3	5 ± 0.3	-543 ± 0.9

whereas 61 %, 67 %, and 81 % were obtained when *A. uvarum* was used. The higher efficiencies obtained with *A. uvarum* for phenolics degradation and decolourization are related with the capacity of this fungus to produce Lac with enzymatic activities of around 200 U/L. Regarding the test carried out with and without solids by *A. uvarum*, no significant differences were observed, so it is not necessary to remove the solids before the biological treatment. Additionally, when pH was initially adjusted to 6, allowing it evolved freely during the treatment, a fast drop of pH occurred (below 3 after 3 day) and sCOD, colour and phenolics removals of 65 %, 94 % and 44 %, were obtained respectively. MnP, LiP and Lac enzymatic activities were notably lower due to the acidification, which was reflected in the lower phenolics degradation obtained in the test without pH control. Nevertheless, pH did not affect sCOD degradation and even allowed a higher decolourization thanks to lignin precipitation. Considering the results obtained in this research, the use of fungi for the treatment of black liquor from the paper industry seems to be a promising option, especially the use of the fungus *A. uvarum*. However, further studies are needed to optimise the process. For example, to evaluate the use of other organic wastes that can be used as a source of nutrients for the fungus or to evaluate the possibility of mixing the black liquor with other industrial effluents. This would avoid the necessity of adding pure reagents such as glucose, or the need to add water to dilute the KBL, which would make the biological treatment process cheaper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (MINECO) through Project CTM2015–63864-R. The authors particularly thank to ENCE Navia (Spain) for their support in the sample collection and to AMG group and Micoteca da University do Minho (MUM) for their useful advice and material support. Ana Isabel Díaz also acknowledges an FPI grant (BES-2016–077963) from Spanish Ministry of Science, Innovation and Universities (MICINN).

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