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Lignin Biodegradation in Pulp-and-Paper Mill Wastewater by Selected White Rot Fungi

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Abstract: An investigation has been carried out to explore the lignin-degrading ability of white rot fungi, as *B. adusta* and *P. crysosporium*, grown in different media containing (i) glucose and mineral salts; (ii) a dairy residue; (iii) a dairy residue and mineral salts. Both fungi were then used as inoculum to treat synthetic and industrial pulp-and-paper mill wastewater. On synthetic wastewater, up to 97% and 74% of lignin degradation by *B. adusta* and *P. crysosporium*, respectively, have been reached. On industrial wastewater, both fungal strains were able to accomplish 100% delignification in 8–10 days, independent from pH control, with a significant reduction of total organic carbon (TOC) of the solution. Results have confirmed the great biotechnological potential of both *B. adusta* and *P. crysosporium* for complete lignin removal in industrial wastewater, and can open the way to next industrial applications on large scale.

Keywords: lignin; delignification; pulp-and-paper-mill c; wastewater; white rot fungi; *B. adusta*; *P. crysosporium*

1. Introduction

The pulp and paper industry in Europe accounts for about a quarter of world manufacturing, producing more than 90 million tons of paper and board, and more than 36 million tons of pulp annually [1]. The manufacture of paper generates significant quantities of wastewater, as high as 60 m^3/ton of paper produced [2]. These raw wastewaters—sometimes called black liquor—can be potentially very polluting [3]. Pulp-and-paper mill wastewater contains a considerable amount of pollutants characterized by high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and high dissolved solids, mainly due to alkali–lignin and polysaccharide degradation residues [4]. The environmental impact of pulp-and-paper mill wastewater depends not only on its chemical nature, but also on its dark coloration that negatively affects aquatic fauna and flora [5]. The primary contributors to the color and toxicity of wastewater are high-molecular-weight lignin and its derivatives. Lignin is the generic term for a large group of aromatic rigid and impervious polymers resulting from the oxidative coupling of 4-hydroxyphenylpropanoids, present predominantly in woody plants [6]. The chemical or biological degradation of lignin is very difficult due to the presence of recalcitrant and not-hydrolysable carbon-carbon linkages and aryl ether bonds [7]. Notwithstanding, pulp-and-paper mills are now facing challenges to comply with stringent environmental regulations [8]. For years, various methods have been developed and attempted for wastewater treatment and organic pollutants removal, including incineration [9], photochemical UV/TiO_2 oxidation [10], adsorption of organic compounds on activated carbon and polymer resin [11], chemical coagulation/flocculation

of lignin using synthetic or natural coagulants [12], and catalytic wet air oxidation [13]. However, all these processes are expensive, environmentally overburdening, and often not very efficient [14]. Furthermore, in these processes lignin is not really degraded, but transferred from a water-suspended state into a solid or absorbed state, only moving the problem [15]. A valid alternative to remove organic pollutants from pulp-and-paper wastewater is now represented by biological treatments. In nature, various ligninolytic organisms and enzymes including fungi, actinomycetes, and bacteria are implicated in lignin biodegradation, and can have potential application in wastewater treatments [16]. Several studies have been carried out on biological delignification of pulp-and-paper mill wastewater using pure bacterial strains [17]: about 70–80% of lignin degradation and COD removal have been achieved with *Pseudomonas putida* and *Acinetobacter calcoaceticus* [18], *Aeromonas formicans* [19], and *Bacillus* sp. [20]. In this field, white-rot fungi have also received increasing attention due to their powerful lignin-degrading enzyme system [21]. White-rot wood fungi use the cellulose fraction as a carbon source and are able to completely degrade the lignin in order to have access to the cellulose. Basidiomycetes species are extensively studied due to the high degradation ability of the extracellular oxidative enzymes (i.e., laccase, peroxidase) that need low-molecular weight cofactors [22]. Recent developments of new technologies and/or improvements of existing ones for the treatment of effluents from the pulp and paper industries include the use of the white rot fungi *Aspergillus foetidus*, *Phanerochaete chrysosporium*, and *Trametes versicolor* [23], but scarce industrial experience is available concerning the degradation of highly-contaminated pulp-and-paper mill wastewater by fungi. In particular, *Phanerochaete chrysosporium* is a well-known white-rot fungus and a strong degrader of various xenobiotics [24]. It has been extensively investigated as a model organism for fungal lignin and organopollutant degradation, since it was the first fungus found to produce lignin peroxidase and manganese peroxidase [25]. *Bjerkandera adusta* is a wood-rotting basidiomycete belonging to the white-rot fungi commonly found in Europe. Its capability to degrade aromatic xenobiotics [26] and extractives [27] has progressively increased its biotechnological interest in wastewater treatments for lignin degradation [28]. Due to its laccase and manganese peroxidase activity [29,30], applications of *B. adusta* to the biomineralization of lignin in soils [31] and to the decoloration of industrial dye effluents [32] has been already attempted, but to date not at an industrial level. This study reports the lignin removal capability and effectiveness of *B. adusta* and *P. crysosporium*, grown in different culture media containing lignin, on synthetic and industrial pulp-and-paper mill wastewater.

2. Materials and Methods

2.1. Fungal Strain Master Cell Bank and Working Cell Bank

Bierkandera adusta and *Phenarochete crysosporium* were purchased from Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The strains have been stored as a master cell bank (MCB), maintained at −20 °C in 3% malt extract and 3% peptone cryovials (1 mL) with added glycerol (0.5 mL). Cells from the MCB were expanded to form the working cell bank (WCB), using an identical procedure. Prior to being used in the process, the fungal strains from WCB were maintained for 7 days in 3% malt extract agar Petri dishes.

2.2. Standard Media and Pulp-and-Paper Mill Wastewater

Three growth media have been prepared for this study: (i) a medium (standard glucose medium, SGM) containing glucose (10 g/L), KH_2PO_4 (1 g/L), yeast extract (0.5 g/L), $MgSO_47H_2O$ (0.5 g/L), and KCl (0.5 g/L) was adjusted to pH 5.5 with 1 M HCl and autoclaved; (ii) a medium (standard lactose medium, SLM) where glucose has been replaced with 50 mL of a dairy residue from cheese processing containing 50 g/L lactose, supplied by Granarolo S.p.A. (Bologna, Italy) (Table 1); (iii) a medium made up with the sole dairy by-product (standard dairy medium, SDM). Before inoculation, SGM medium was added with 5 g/L of standard lignin. Spore and mycelium suspensions obtained from agar Petri dishes were used to inoculate a 250 mL Erlenmeyer flask containing 100 mL of SGM. Cell cultures were

all incubated at 24 °C without pH control for 10 days under mild stirring rate (60 rpm) and samples were withdrawn at 1–3 day intervals for residual lignin content analysis.

Table 1. Dairy residue chemical composition.

Constituent	%
Total solids	6.0
Lactose	5.0
Proteins	0.6
Non-protein N *	0.2
Lipids	0.05
Ash	0.5

Note: * N = Nitrogen.

A synthetic pulp-and-paper mill wastewater was prepared by dissolving 5 g/L of standard lignin in distilled water. Three 1-L Erlenmeyer flasks containing 500 mL of the synthetic wastewater were inoculated with 50 mL of cell cultures grown in the SGM, SLM, and SDM media, respectively, all added with standard lignin (5 g/L) and incubated for 10 days at 24 °C and mild agitation (60 rpm).

The industrial pulp-and-paper mill wastewater utilized for this study was supplied by a local pulp-and-paper firm, collected in a closed container and stored in darkness at 4 °C until use. The concentration of soluble and insoluble lignin was determined, as well as total organic carbon (TOC), as described in Section 2.3.

Two 1-L Erlenmeyer flasks containing 500 mL of wastewater were inoculated with 50 mL of cell cultures grown in the SLM added with lignin (5 g/L), and incubated for 10 days at 24 °C and mild agitation (60 rpm). In one flask, pH was adjusted to 5.5 with 1 M HCl, in the other pH was left at the original value measured for industrial wastewater of 6.5 without control.

All of the above experiments were conducted in triplicate. The data in subsequent sections are based on the average and standard deviation of the three measurements.

2.3. Chemicals and Analysis

All chemicals were reagent grade or better. Unless specified otherwise, they were obtained from Sigma-Aldrich Chemical Co (Saint Louis, MO, USA). The concentration of lignin was measured using the INNVENTIA—Biorefinery Test Methods L 2:2016 [33], specific for the determination of lignin isolated from a Kraft pulping process. The procedure is based on the sulphuric acid hydrolysis of the samples. This method makes it possible to determine concentrations of total lignin content, measured as the sum of the amount of acid-insoluble matter (AIM) and acid-soluble matter (ASM) after sulphuric acid hydrolysis, down to 10 mg/g oven-dry sample.

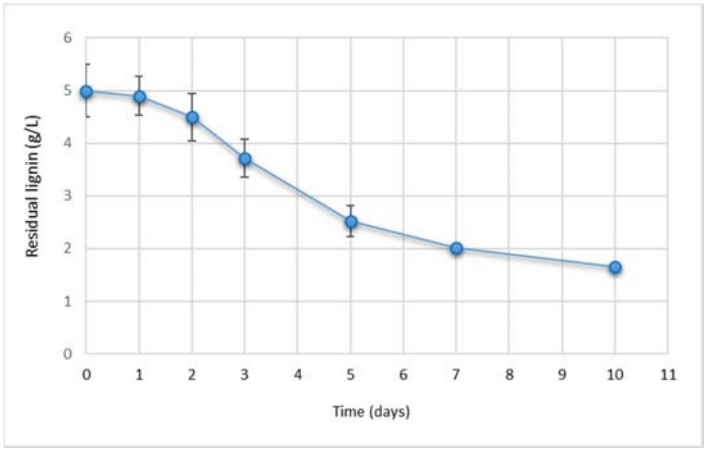
TOC was determined with a Carbon Analyzer TOC-V-CSM (Shimadzu, Tokio, Japan) after acidification with 2 M HCl to remove dissolved carbonate [34]. The instrument has a detection limit of 5 μg/L and a measurement accuracy expressed as coefficient of variation (CV) 1.5%. Biomass concentration (dry weight, DW) was determined gravimetrically after drying overnight at 105 °C on a pre-weighed 0.2 μm filter.

3. Results

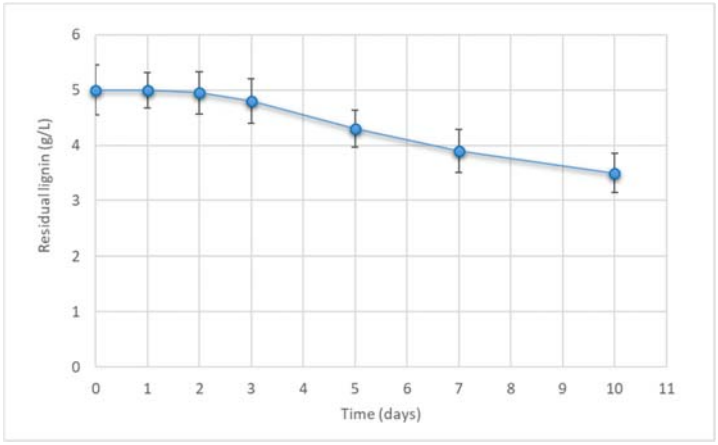
3.1. B. adusta and P. crysosporium Growth on SGM

Lignin was added to the standard medium SGM before inoculation of *B. adusta* and *P. crysosporium* because several studies describe that the presence of lignin in the liquid medium exerts an influence on the expression profile of lignin peroxidase, manganese peroxidase, and laccase—all enzymes held responsible for the lignin degradation of natural lignocellulosic residue [35,36]. Under the condition

maintained on 100 mL-scale, in 10 days *B. adusta* was able to uptake and metabolize lignin up to 67%, while *P. crysosporium* only 30% (Figure 1).



(**a**)



(**b**)

Figure 1. Lignin removal from 100 mL of standard glucose medium (SGM) with lignin 5 g/L added by (**a**) *B. adusta* and (**b**) *P. crysosporium.*

As described by Girard et al. [37], in both cases the expression of delignifying enzymes only initiated after 2–3 days from inoculation, corresponding to complete glucose depletion (data not shown).

3.2. Lignin Removal Efficiency on Synthetic Pulp-and-Paper Mill Wastewater

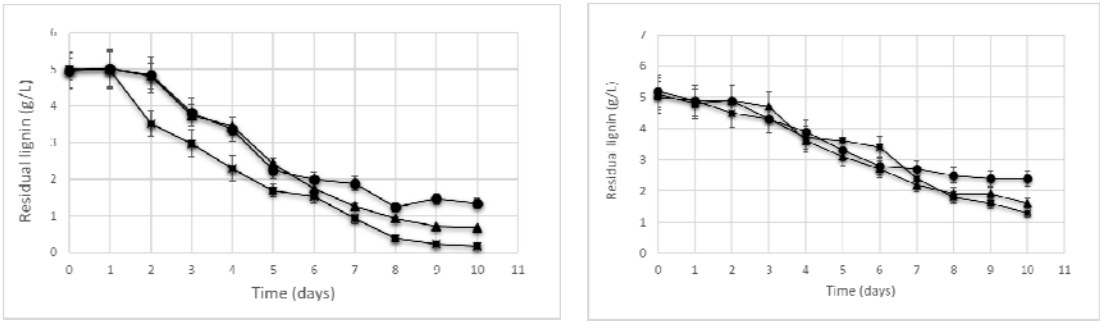
The addition of agro-food by-products to fungal cultures may reflect complex growth conditions close to nature, and could stimulate the secretion of various enzymes required for degradation or detoxification processes [38]. This, in addition to the evidence that the production of lignin peroxidase and manganese peroxidase in *B. adusta* is stimulated by the presence of organic nitrogen (N) source (unlike *P. chrysosporium*, which produces ligninolytic peroxidases in response to N limitation [39]), has driven the study towards the possibility of integrating the growth medium with a dairy by-product, usually rich in protein and amino acids, apart from sugar. Furthermore, in view of industrial application, the use of a by-product instead of pure substrates could permit the considerable reduction of operational investments, among which chemicals required for fungal growth are the most relevant. The use of cheese whey has been previously proposed by Feijoo et al. [40] as an inexpensive substrate for fungal growth. *B. adusta* and *P. chrysosporium* have been incubated in SGM, SLM, and only dairy

residue with no addition of other nutrients or mineral salts (SDM). The largest amount of fungal biomass was obtained when dairy residue was present in the media (Table 2).

Table 2. Fungal cells dry weight (g/L) obtained from growth in SGM, standard lactose medium (SLM), and standard dairy medium (SDM) media.

Strain	SGM	SLM	SDM
B. adusta	2.5 ± 0.4	3.6 ± 0.5	3.5 ± 0.4
P. crysosporium	2.7 ± 0.3	4.3 ± 0.5	3.8 ± 0.6

In both cases, the results seem to confirm the correlation between organic N source and fungal cell growth. Identical amounts of cells of *B. adusta* and *P. crysosporium* grown in the three media were used as inoculum for synthetic wastewater, in order to verify if cell cultures developed in different media would express different enzymatic patterns or different enzyme activities. Figure 2a shows that *B. adusta* grown in the SGM medium was able to remove 73% of lignin, whilst *B. adusta* grown in the presence of a source of protein and amino acids in both cases reached delignification yields of 97% with SLM and 86% with SDM. On the other hand, *P. crysosporium* in all three cases obtained yields not higher than 74% when grown in SLM (54% in SGM and 69% in SDM, respectively).



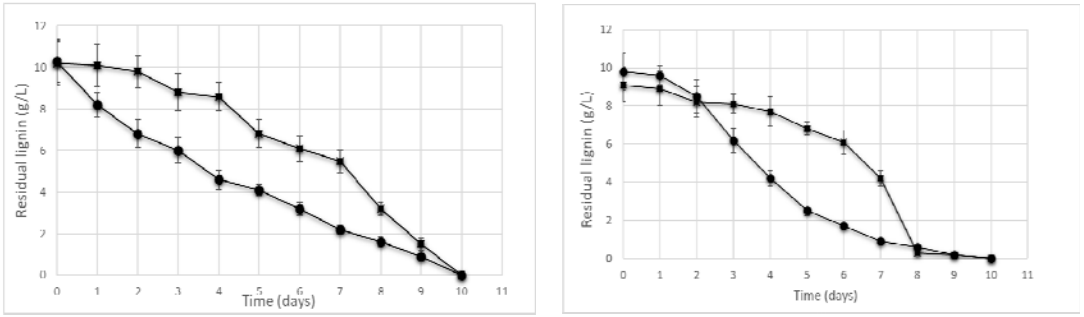
(**a**) (**b**)

Figure 2. Lignin removal from synthetic pulp-and-paper mill wastewater during 10 days by (**a**) *B. adusta* and (**b**) *P. crysosporium* grown in SGM (dots), SDM (triangles), and SLM (squares) media, all with the addition of 5 g/L lignin.

The time courses of delignification in 10 days were quite similar in all three cases for *B. adusta*, having a 1-day reduced lag phase cell culture grown in SLM. The interesting point is that the slopes of the three curves are similar in the 3–8 days' interval, but from day-8 on, cell culture grown in SGM seemed to miss the lignin removal capacity, even though residual lignin was still present in the fermentation broth. This could be due to the decline of lignin peroxidase activity caused by the appearance of extracellular protease activity that has been observed after day 6–10 in cultures of *P. chrysosporium* grown on glucose [41]. This also confirmed what was reported by Nakamura et al. [42], whereby in glucose-based media, enzymes produced by *B. adusta* can only degrade part of the chemical structure of lignin. Otherwise, in order to maximize peroxidase activity, lactose has already been identified as a good carbon source for *Bierkandera* spp. when the nitrogen source was organic [43], as in SLM and SDM media. *P. crysosporium* was found to be surprisingly less active than *B. adusta* in lignin removal effectiveness in all three growth conditions (Figure 2b). Moreover, it showed a longer lag phase before starting to degrade lignin. According to Keyser et al. [44], lignin metabolism in *P. crysosporium* did not reflect the depletion of glucose, as in *B. adusta*, but instead appeared to be a response to nitrogen starvation. The prolonged lag phase could be induced by the need to wait for the partial or complete depletion of the N source transferred with inoculum.

3.3. Lignin Removal Efficiency on Industrial Pulp-and-Paper Mill Wastewater

B. adusta grown on SLM with lignin 5 g/L added has demonstrated to be effective for almost complete lignin biodegradation in synthetic wastewater. Based on these promising results, an application on industrial wastewater has been attempted, in comparison with *P. crysosporium* grown in the same conditions. The industrial wastewater supplied by the local pulp-and-paper mill for these tests (pH 6.5 and with a 100 g/L lignin content on dry weight basis) was diluted (12% dry weight). The ability of both fungal strains to biodegrade lignin has been tested, verifying the effect of pH on their enzymatic activities. In one case, the pH of wastewater was adjusted to the optimum value for fungi cell growth (pH 5.5), and in the other the process was allowed to proceed without correction (pH 6.5). From the perspective of industrial application, the possibility of avoiding costs deriving from the use of acids as correction agent could be very relevant. The results of the tests carried out using an inoculum of *B. adusta* and *P. crysosporium* grown on SLM medium on industrial wastewater with and without pH correction are reported in Figure 3.

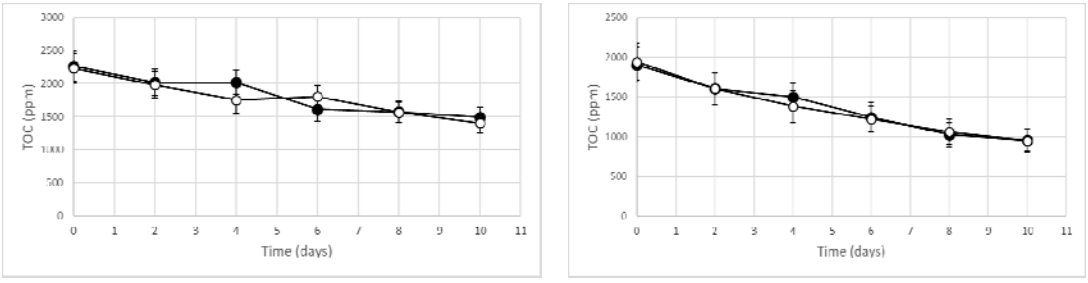


(**a**) (**b**)

Figure 3. Lignin removal from industrial pulp-and-paper mill wastewater with pH correction (dots) and without pH correction (squares) during 10 days by (**a**) *B. adusta* and (**b**) *P. crysosporium*, grown in SLM medium added with lignin 5 g/L.

As expected, at optimum pH condition *B. adusta* started to biodegrade lignin without any lag phase and maintained an almost constant biodegradation rate of about 1 g/L of lignin per day over the entire test course. In contrast, without pH control fungal cells needed 1–2 days for adapting, before starting biodegradation. This leads to a variable delignification rate during the process—slower at the beginning (0.9 g/L × day) and higher from five days on (1.7 g/L × day). The final result in both cases was complete lignin removal, with an efficiency of 100%. One hundred percent delignification was also obtained when treating the pulp-and-paper mill wastewater with *P. crysosporium*, almost complete in 8 days. At a first glance, the time courses seemed to confirm the previous results obtained on synthetic pulp-and-paper mill wastewater, regarding the need of a longer lag phase compared with *B. adusta*. Otherwise, a sharp decline of residual lignin was observed from day 6. These results appeared to be particularly promising, compared with an average delignification yield of 70–80% reported for white rot fungi: both *P. crysosporium* and *B. adusta* were competitive against the 71% delignification yield on pulp-and-paper mill residues obtained by *Pseudomonas putida* [45], 78% by *Aeromonas formicans* [19], and 80% by *Acinetobacter calcoaceticus* [46].

To confirm the overall organic C removal, TOC analysis of samples was carried out. It is usually reported that lignin represents about 30–45% of the total organics in pulp-and-paper mill wastewater [47], so a corresponding decrease of TOC was expected (Figure 4). In both cases, an overall reduction of about 35% of organic charge of wastewater was obtained, reasonably due to lignin uptake for fungal metabolism.



(**a**) (**b**)

Figure 4. Total organic carbon (TOC) decrease of industrial pulp-and-paper mill wastewater with pH correction (white dots) and without pH correction (black dots) during 10 days by (**a**) *B. adusta* and (**b**) *P. crysosporium* grown in SLM medium with 5 g/L lignin added.

4. Conclusions

This study opens new perspectives for the bioremediation of industrial effluents such as pulp-and-paper mill wastewater using white rot fungi. In particular both *B. adusta* and *P. crysosporium* were found able to growth on non-conventional media, better than on glucose as sole carbon source, and to improve the delignifying activity in the presence of organic N and mineral salts. Moreover, they can survive on synthetic wastewater and proved to be effective for the complete degradation of lignin. The biotechnological potential of these strains was also confirmed on industrial wastewater, being active up to the total depletion of lignin. No operational problem was detected at 500 mL scale, as a first confirmation of the robustness and applicability of this system. The results obtained lay the ground for further scaling up to pilot plant level.

Author Contributions: Davide Gavino Dedola, Riccardo Blo and Irene Rugiero performed all the experiments and carried out all the analytical assays, also giving a great contribution to the discussion. Simone Pellizzari conceived and designed the experiments, together with Stefania Costa and Elena Tamburini, who wrote the manuscript. As supervisor of the research group, Paola Pedrini defined the general research statement.

Conflicts of Interest: The authors declare no conflict of interest.

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