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Lignin biodegradation in pulp-and-paper mill wastewater by selected white rot fungi

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Abstract: An investigation was carried out to explore the lignin-degrading ability of white rot fungi, as *B.adusta* and *P.crysosporium*, grown in different media containing glucose as carbon source and mineral salts, a dairy residue containing lactose and organic N and a mixture of dairy residue and mineral salts. Both fungi were then used as inoculum to treat synthetic and industrial black liquor. Up to 97% and 74% of lignin degradation in synthetic black liquor have been obtained respectively by *B.adusta* and *P.crysosporium* grown on dairy residue added with mineral salts. On industrial black liquor, 100% of delignification have been accomplished by both fungal strains in 8-10 days with or without pH control, and a significant effect on total organic carbon (TOC) reducing. Results have confirmed the great biotechnological potential of both *B.adusta* and *P.crysosporium* for complete lignin removal in industrial black liquor and can open the way to industrial application.

Keywords: lignin, delignification, pulp and paper mill, wastewater, black liquor, 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]. Such raw wastewaters, usually called black liquors, can be potentially very polluting [3]. The black liquor contain 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 black liquor 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 black liquor are high-molecular-weight lignin and its derivatives. As is known, 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 the wood plant [6]. The chemical or biological lignin degradation 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 black liquor treatment and organic

pollutants removal, as 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 cost expensive, environmentally overburdening and often not very efficient [14]. Furthermore, in this 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 black liquor treatments [16]. Several studies have been carried out on biological delignification of black liquor 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 also white-rot fungi have received a rising attention due to their powerful lignin-degrading enzyme system [21]. White-rot wood fungi use the cellulose fraction as of carbon source and are able to degrade completely the lignin 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 ~~in~~ of new technologies and/or improvement 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 few industrial experiences are available concerning the degradation by fungi of highly contaminate black liquors. In particular, *Phanerochaete chrysosporium* is a well-known white-rot fungus and a strong degrader of various xenobiotics [24]. It has been to date 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 also for lignin degradation [28]. Due to its laccase and manganese peroxidase activity [29,30], applications of *B. adusta* to biomineralization of lignin in soils [31] and to decoloration of industrial dye effluents [32] has been already attempted, but at the best Authors' knowledge to date not at 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 black liquor.

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 Master Cell Bank (MCB), maintained at -20°C in 3% malt extract and 3% peptone cryovials (1 ml) added with glycerol (0.5 ml). Cells from the MCB were expanded to form the Working Cell Bank (WCB), using identical procedure. Prior to be 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 black liquor

Three growth media have been prepared for this study: i) a medium (SGM) containing glucose, 10 g/L, KH_2PO_4, 1 g/L, yeast extract, 0.5 g/L, $MgSO_4x7H_2O$, 0.5 g/L, KCl, 0.5 g/L was adjusted to pH 5 with 1M HCl and autoclaved; ii) a medium (SLM) where glucose has been replaced with 50 ml of a dairy residues from cheese processing containing 50 g/L lactose, supplied by Granarolo S.p.A. (Bologna, Italy); iii) a medium made up with the sole dairy by-product (SDM). Before inoculum, SGM medium has been added with 5 g/L of standard lignin. Spore and mycelium suspension obtained from agar Petri dishes were used to inoculate a 250 ml Erlenmeyer flask containing 100 ml of SGM medium. Cell cultures have been all incubated at 24°C without pH control for 10 days under

mild stirring rate (60 rpm) and samples withdrawn at 1-3 days interval for residual lignin content analysis.

A synthetic black liquor has been prepared dissolving 5 g/L of standard lignin in distilled water. Three 1-liter Erlenmeyer flasks containing 500 ml of the synthetic black liquor 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 black liquor utilized for this study was supplied by local pulp and paper firm, collected in a closed container and stored in obscurity at 4 °C until use. The concentration of soluble and insoluble lignin has been determined, as well as TOC.

Two 1-liter Erlenmeyer flasks containing 500 ml of black liquor 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 1M HCl, in the other pH was left at original value of 6.5 without control.

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

2.3 Chemicals and Analysis

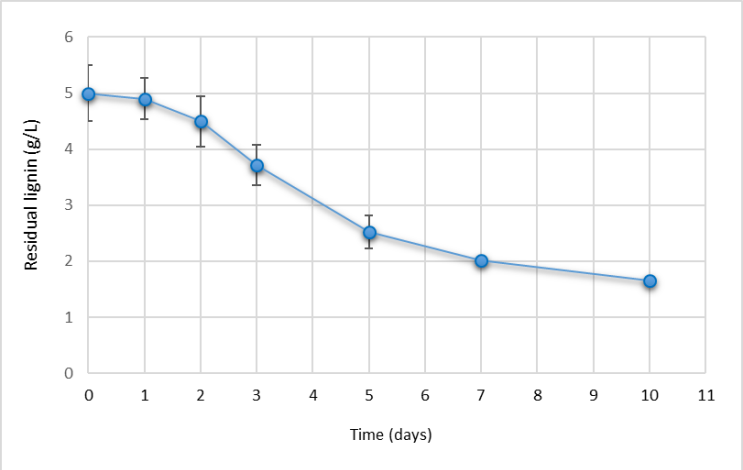
All chemicals were reagent grade or better. Unless specified otherwise, they were obtained from Sigma-Aldrich Chemical Co. (Cincinnati, OH, USA). The concentration of lignin was measured using the INNVENTIA - Biorefinery Test Methods L 2:2016 [33], specific for the determination of lignin isolated from 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 has been 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 CV 1.5%. Biomass concentration (dry weight, DW) was determined gravimetrically after drying it overnight at 105°C on a pre-weighed 0.2 µm filter (Millipore, Billerica, MA, USA).

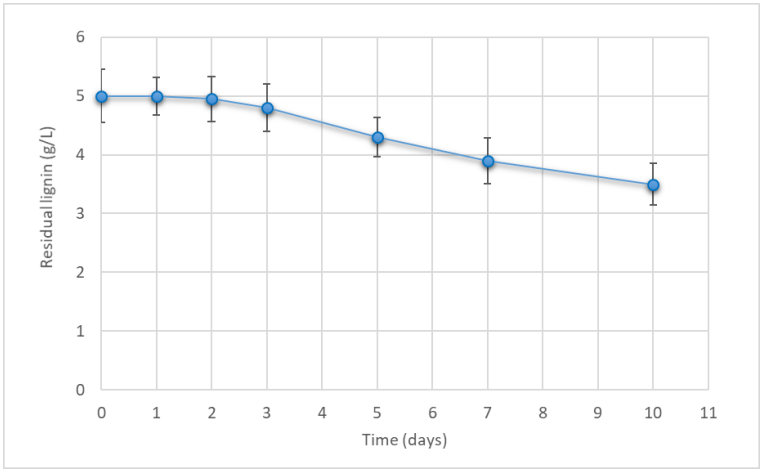
3. Results

3.1 B. adusta and P.crysosporium growths on SGM

The standard medium SGM was added of lignin to 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 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 SGM medium added with lignin 5 g/L by (a) *B. adusta* and (b) *P. crysosporium.*

As expected [37], dignifying enzymes pattern in both cases initiated to be expressed only after 2-3 days from inoculum, corresponding to the complete glucose depletion (data not shown).

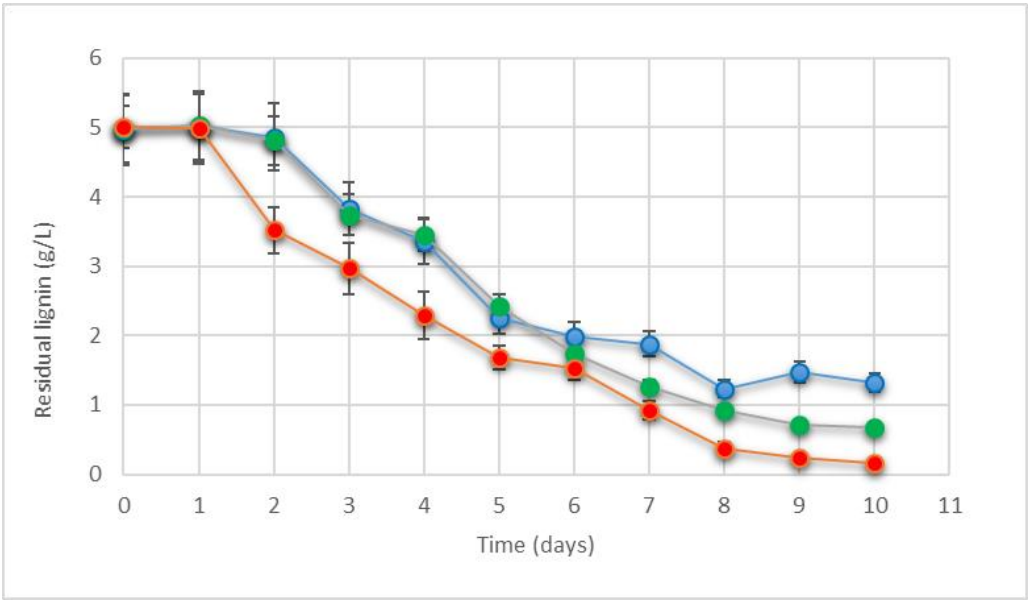
3.2 Lignin removal efficiency on synthetic black liquor

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 N source, unlike *P. chrysosporium* which produces ligninolytic peroxidases in response to N limitation [39], has driven the study towards the possibility to integrate the growth medium with a dairy by-product, usually rich in protein and aminoacids, apart from sugar. Furthermore, in view of industrial application, the use of a by-product instead of pure substrates could permit to considerably reducing 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 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 has been obtained when dairy residue was present in the media (Table 1).

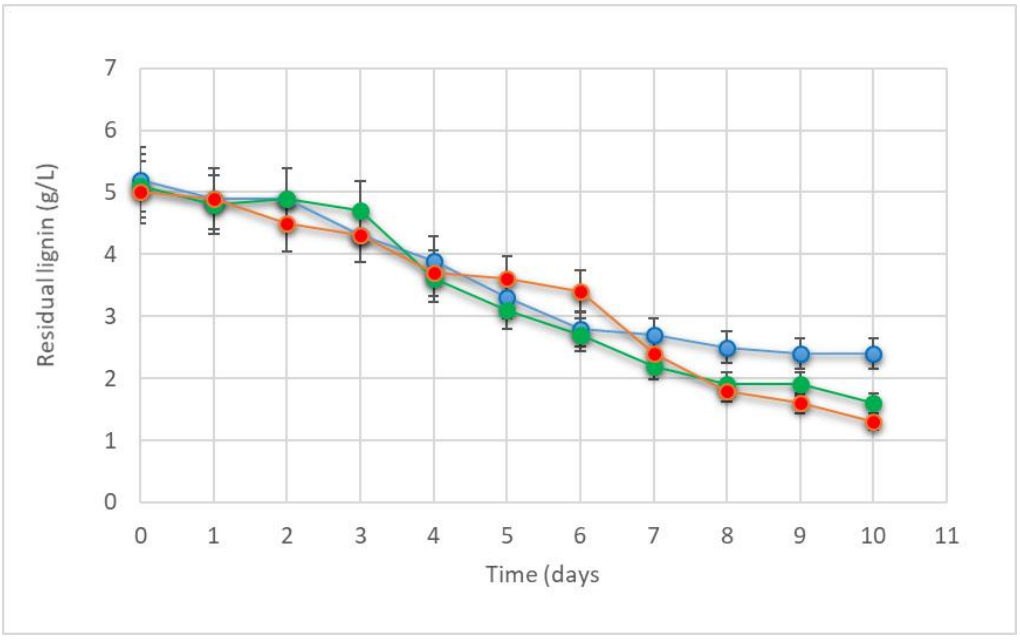
Table 1. Fungal cells dry weight (g/L) obtained from growth in SGM, SLM and SDM media.

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

It seems to confirm in both cases the correlation between organic N source and fungal cell growth. An identical amount of cells of *B. adusta* and *P. crysosporium* grown in the three media has been used as inoculum for synthetic black liquor, in order to verify if cell cultures developed in different media would express different enzymatic patterns or different enzyme activities. Figure 2(a) 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 aminoacids has reached in both cases yields of delignification of 97% with SLM and 86% with SDM. On the other hand, *P. crysosporium* in all the three cases have obtained yields not higher than 74% when grown in SLM (54% in SGM and 69% in SDM, respectively).



(a)



(b)

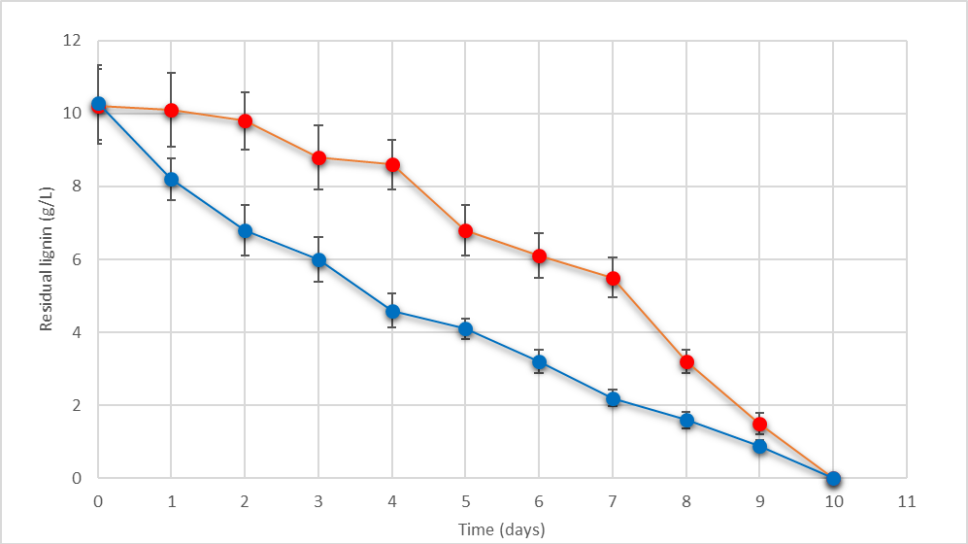
Figure 2. Lignin removal from synthetic black liquor by (a) *B.adusta* and (b) *P.crysosporium* grown in SGM (blue); SDM (green) and SLM (red) media, all added with lignin 5g/L.

The time courses of delignification in 10 days were quite similar in all the three cases for *B. adusta*, having a 1-day reduced lag phase cell culture grown in SLM. The interesting point is that the slope 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 a residual of 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 have 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 been already identified as a good carbon source for *Bierkandera* spp. when the nitrogen source was organic [43], as in SLM and SDM media. *P. crysosporium* has been found surprisingly less active than *B.*

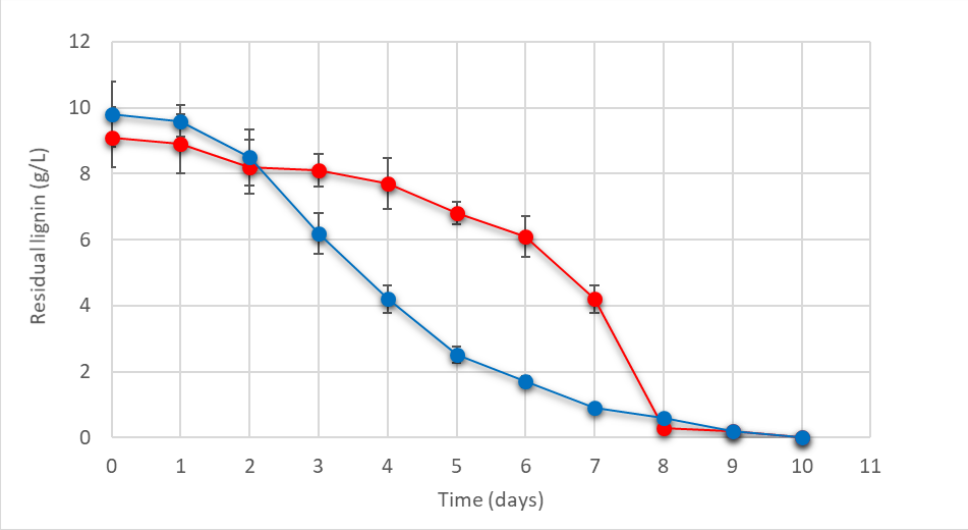
adusta in lignin removal effectiveness in all the three conditions of growth (Figure 2(b). Moreover, it showed a longer lag phase before starting to degrade lignin. According to Keyser et al. [44], in *P. crysosporium* lignin metabolism did not reflect depletion of glucose, as in *B.adusta*, but instead appeared to be a response to nitrogen starvation. The prolonged lag phase could be induced by the need to wait the partial or complete depletion of the N source transferred with inoculum.

3.3 Lignin removal efficiency on industrial black liquor

B. adusta grown on SLM added with lignin 5 g/L has demonstrated to be effective for almost completely lignin biodegradation in synthetic black liquor. Based on this promising results, an application on industrial black liquor has been attempted, in comparison with *P.crysosporium* grown in the same conditions. The industrial black liquor supplied by the local pulp and paper mill for these tests (pH 6.5 and with a 10% 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 enzymes activities. In one case the pH of black liquor was adjusted to the optimum value for fungi cell growth (pH 5.5) and in the other was let without correction (pH 6.5). In a perspective of industrial application, the possibility to avoid 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 black liquor with and without pH correction have been reported in Figure 3.



(a)

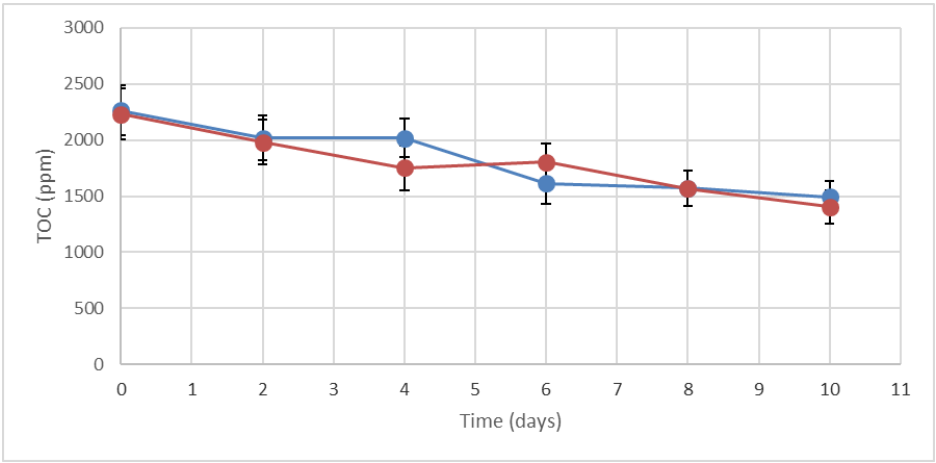


(b)

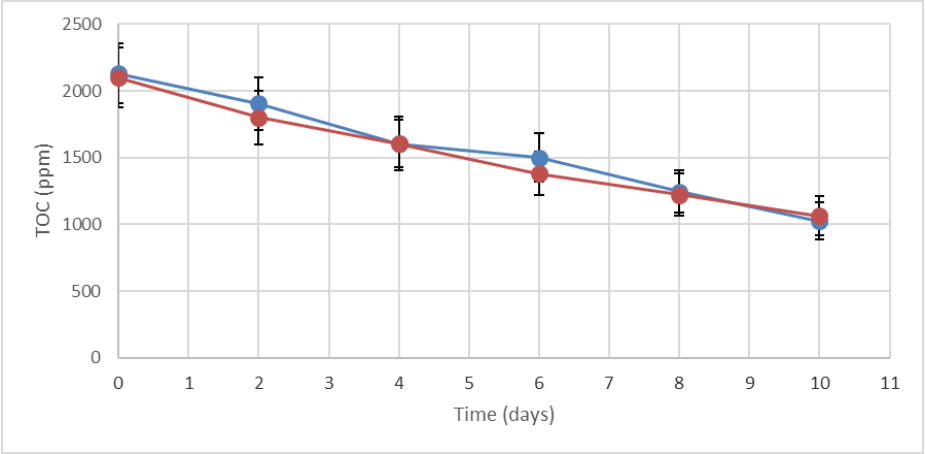
Figure 3. Lignin removal from industrial black liquor with pH correction (blue) and without pH correction (red) by (a) *B.adusta* and (b) *P.crysosporium*, grown in SLM medium added with lignin 5g/L.

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. Differently, without pH control fungal cells need 1-2 days for adapting, before starting biodegradation. This leads to a not constant 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 case was the complete lignin removal with an efficiency of 100%. 100% of delignification was also obtained treating the black liquor with *P. crysosporium*, almost complete in 8 days. At a first glance, the time courses seemed to confirm the previous results obtained on synthetic black liquor, regarding to the need of a longer lag phase compared with *B. adusta*. Otherwise, from day 6 a sharp decline of residual lignin was observed. This results appeared particularly promising, compared with an average delignification yield of 70-80% reported in literature for white rot fungi. Both *P.crysosporium* and *B. adusta* were competitive against 71% of delignification yield on pulp and paper mill residues obtained by *Pseudomonas putida* [45], 78% by *Aeromonas formicans* [46] and 80% by *Acinetobacter calcoaceticus* [47].

To confirm the overall organic C removal, TOC analysis of samples has been carried out. It is usually reported that lignin represents about 30-45% of the total organics in black liquors [48], so a corresponding decreasing of TOC have been expected (Figure 4). In both case, an overall reduction of about 35% of organic charge of black liquor has been obtained, reasonably due to lignin uptake for fungi metabolism.



(a)



(b)

Figure 4. TOC of industrial black liquor with pH correction (blue) and without pH correction (red) by (a) *B.adusta* and (b) *P.crysosporium* grown in SLM medium added with lignin 5g/L.

4. Conclusions

This study opens new perspectives for the bioremediation of industrial effluents 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 the sole glucose as carbon source and to improve the delignifying activity in the presence of organic N and mineral salts. Moreover, they can survive on synthetic black liquor and proved to be effective for the complete degradation of lignin. The biotechnological potential of theses strain was confirmed also on industrial black liquor, being active up to the total depletion of lignin. No operational problem was detected at 500ml scale, as a first confirmation of the robustness and applicability of this system. The results obtained lay the ground to 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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