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Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm

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Abstract

An investigation was conducted to explore the lignin-degrading capacity of attached-growth white-rot fungi. Five white-rot fungi, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Lentinus edodes*, *Trametes versicolor* and S22, grown on a porous plastic media, were individually used to treat black liquor from a pulp and paper mill. Over 71% of lignin and 48% of chemical oxygen demand (COD) were removed from the wastewater. Several factors, including pH, concentrations of carbon, nitrogen and trace elements in wastewater, all had significant effects on the degradation of lignin and the removal of COD. Three white-rot fungi, *P. chrysosporium*, *P. ostreatus* and S22, showed high capacity for lignin degradation at pH 9.0–11.0. The addition of 1 g l^{-1} glucose and 0.2 g l^{-1} ammonium tartrate was beneficial for the degradation of lignin by the white-rot fungi studied.

Keywords: Black liquor; Biodegradation; Lignin; COD; White-rot fungi

1. Introduction

The pulp and paper industries produce large quantities of toxic and intensely colored waste effluents, causing severe water pollution. This kind of effluent, usually called black liquor, has a high level of chemical oxygen demand (COD). The primary contributors to the color and toxicity of these effluents are high-molecular-weight lignin and its derivatives. The ^{13}C-NMR analysis illustrates the presence of β-*O*-4 ether bonds, β-5 and 5-5′ carbon–carbon linkages between the lignin molecules, which are not hydrolysable (Sun et al., 1999). As a result, lignin is very difficult to degrade either chemically or biologically.

For several decades, a number of methods for black liquor treatment have been developed, e.g., adsorption of organic pollutants from kraft pulp mill wastewater using activated carbon and polymer resin (Zhang and Karl, 2001), and chemical coagulation of lignin from pulp and paper wastewater using synthetic and natural coagulants (Ganjidoust et al., 1997). However, these processes are not very effective, but are costly. Furthermore, in these processes lignin compounds are not degraded, but are just transferred from a water-soluble state into a solid state.

Conventional aerobic and anaerobic treatment methods have been found to be able to partially remove COD from pulp and paper wastewater. Both activated sludge and anaerobic digestion processes have been employed for the treatment of pulp and paper mill effluent (Thompson et al., 2001). The combination of physicochemical and biological treatment has also been tested to explore the technical feasibility. For instance, a combination of ozone and activated sludge process has been found to be able to treat kraft pulp wastewater efficiently (Nakamura et al., 1997). In the studies concerning the

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treatment of pulp and paper wastewater, the biodegradation of lignin has been a focus (Tuomela et al., 2000; Leonowicz et al., 1999).

Several researchers have investigated biological treatment of black liquor by using various pure bacterial strains. The degradation efficiency of lignin in black liquor was 70–80% and COD removal efficiencies of 70–80% were achieved with *Pseudomonas putida* (Srivastava et al., 1995) and *Acinetobacter calcoaceticus* (Jain et al., 1997). In a batch test on the treatment of black liquor from a kraft pulp and paper mill by *Aeromonas formicans*, around 71% of COD and 78% of lignin were removed (Gupta et al., 2001).

In addition to bacteria, white-rot fungi have received extensive attention due to their powerful lignin-degrading enzyme system (Livernoche et al., 1983; Reid et al., 1985; Frederick et al., 1991). *Aspergillus foetidus* was studied for its ability to remove color, COD and lignin from bagasse-based pulp and paper mill wastewater (Sumathi and Phatak, 1999). Nearly 90–95% of the total color was removed, and the simultaneous reduction in color and lignin level indicated a strong correlation between the decolorization and lignolytic processes. The degradation abilities of *Oxysporus* sp., *Phanerochaete chrysosporium* and *Schizophyllum commune* were evaluated for the removal of lignin from olive pomace (Haddadin et al., 2002). Among the three fungi, *Oxysporus* sp. was the most effective for lignin degradation with an efficiency of 70%, whereas *P. chrysosporium* achieved lignin removal of 60%.

Previous work has focused mainly on the decolorization of black liquor and the degradation of lignin by suspended-growth white rot fungi (Garcia et al., 1987; Kerem et al., 1992). Information about lignin degradation and COD removal by suspended-growth white rot fungi is still sparse. Therefore, the main objective of the present work was to compare the lignin-degrading abilities of five white rot fungi, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Lentinus edodes*, *Trametes versicolor* and S22, on the supporting media, and to investigate the effects of pH, concentrations of carbon, nitrogen and trace elements in the wastewater medium on the removal of lignin and COD.

2. Methods

2.1. Fungi

Five strains of white-rot fungi were used in this study. *Phanerochaete chrysosporium* was obtained from the Institute of Microbiology, Guangzhou, China. *Pleurotus ostreatus*, *Lentinus edodes*, and *Trametes versicolor* were purchased from the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. The strain of S22 was isolated and screened by the Laboratory of Microbiology, Anhui University, China. All these strains were maintained in 2% malt extract agar slants at 4 °C.

2.2. Medium and the treatment of black liquor

The raw black liquor, with initial COD and lignin concentrations of 23.0 g l^{-1} and 4.6 g l^{-1}, respectively, was obtained from Dongtai Paper Company, Anhui, China. This raw liquor was diluted with distilled water to a concentration of 20%, resulting in COD and lignin concentrations of 4.6 g l^{-1} and 0.92 g l^{-1}, respectively, for the tests. Spore suspensions or mycelial suspensions obtained from agar slants were used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of 20% black liquor solution. The solution, called wastewater medium, also contained KH_2PO_4 of 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ of 0.5 g l^{-1}. Glucose was used as the carbon source, and ammonium tartrate was used as the nitrogen source. Porous plastic rings were used as supporting media for the attached growth of fungi. The plastic rings were 0.7 cm in height and 1 cm in internal diameter. They had a density of 0.96 g ml^{-1}, specific surface area of 399 $m^2 m^{-3}$ and porosity of 83%. The pH value of the wastewater medium was adjusted with 1 N NaOH or 1 N HCl before sterilization, and 50 plastic rings were placed in each flask and sterilized at 121 °C for 30 min. Incubation was carried out without agitation for 16 days at 28 °C. The concentration of lignin and COD was determined at intervals as determined.

The above experiments were conducted in triplicate. A flask containing wastewater but no white-rot fungus was also run in parallel as a control. The data in subsequent sections are based on arithmetic means of three measurements.

2.3. Analysis

The concentration of lignin in wastewater was measured using the method proposed by Lundquist et al. (1977). The measurement was carried out by using a UV–Vis spectrophotometer (UV752-GD, Shanghai Analytical Instrument Co.) at 280 nm. The COD was determined according to standard methods (APHA, AWWA and WEF, 1992).

3. Results and discussion

3.1. Comparison of five white-rot fungi for the removal of lignin and COD

The removal of lignin and COD from wastewater medium by five white-rot fungi is shown in Fig. 1 as a function of incubation time. The COD and lignin concentrations were analyzed after a 4-day incubation, as previous work demonstrated that ligninolytic enzymes, including LiP,

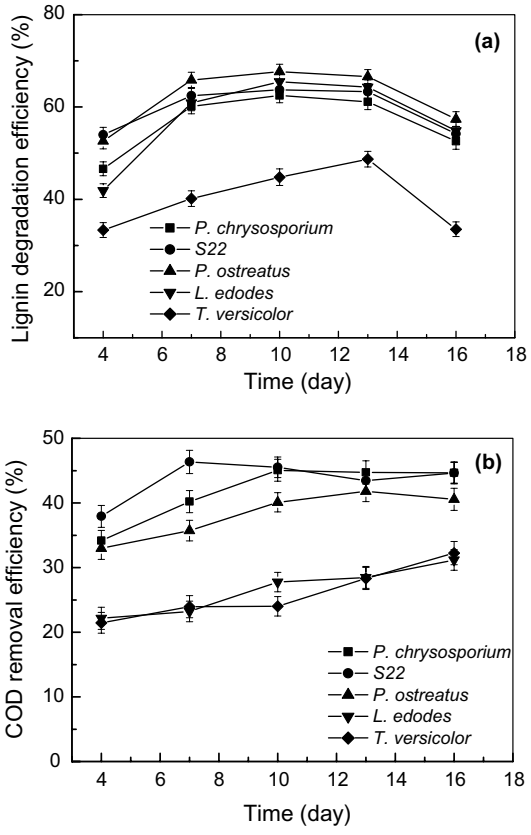

Fig. 1. Comparison of five white-rot fungi for the removal of lignin and COD from wastewater medium with glucose 1 g l^{-1}, ammonium tartrate 0.2 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1} and pH 6.0. Initial lignin concentration: 0.92 g l^{-1}, COD: 4.58 g l^{-1}. (a) Lignin degradation efficiency; (b) COD removal efficiency.

MnP and laccase, were detected after four days of inoculation (Wu et al., 2002). This result was in good agreement with that reported by Frederick et al. (1991).

Among the five strains tested, *P. chrysosporium*, *P. ostreatus* and S22 demonstrated higher capabilities for degrading lignin than *Lentinus edodes* and *Trametes versicolor* (Fig. 1a). The initial degradation of lignin was fast, reaching 54% for S22 and 52% for *P. ostreatus* on day 4. Thereafter, the degradation of lignin slowed down, achieving 60% removal efficiency on day 7. This result shows that there were slower changes in the degradation of lignin with time going on, probably due to cell autolysis or the depletion of glucose in the culture (Frederick et al., 1991). For *L. edodes*, lignin removal efficiency of 65% was achieved on day 10. The highest removal efficiency of lignin was only 49% with *T. versicolor* on day 13, indicating that this fungus had the slowest lignin degradation rate.

Effective reduction of COD was observed for *P. chrysosporium*, *P. ostreatus* and S22. Over 46% of COD was removed by S22 in the 7-day incubation, while maximum COD removal efficiency was found for both *P. chrysosporium* and *P. ostreatus* on day 10. However, a low COD removal efficiency of 30% was obtained with *L. edodes* and *T. versicolor*. There was no significant increase in COD removal efficiency with the increase in incubation time until day 16. For all five fungi, COD removal coincided with lignin degradation.

It was also observed that the formation of biofilm was faster with *P. chrysosporium*, *P. ostreatus* and S22 than with *L. edodes* and *T. versicolor*. This was in accord with the higher removal efficiencies of lignin and COD for the former three fungi than for the latter two fungi.

3.2. Effect of carbon source concentration

The above experimental results demonstrated that, among the five white-rot fungi, *P. ostreatus* had the highest lignin degradation efficiency (68%). Thus, this fungus was chosen to evaluate the influences of nutrients on the lignin degradation.

Under the conditions of the presence and absence of the trace elements, the removals of lignin and COD by *P. ostreatus* at pH 6.0 were compared at various glucose concentrations. As shown in Fig. 2a, at a glucose concentration of 1 g l^{-1}, the lignin removal efficiency was 66% on day 7 and 68% on day 10, whereas at a glucose

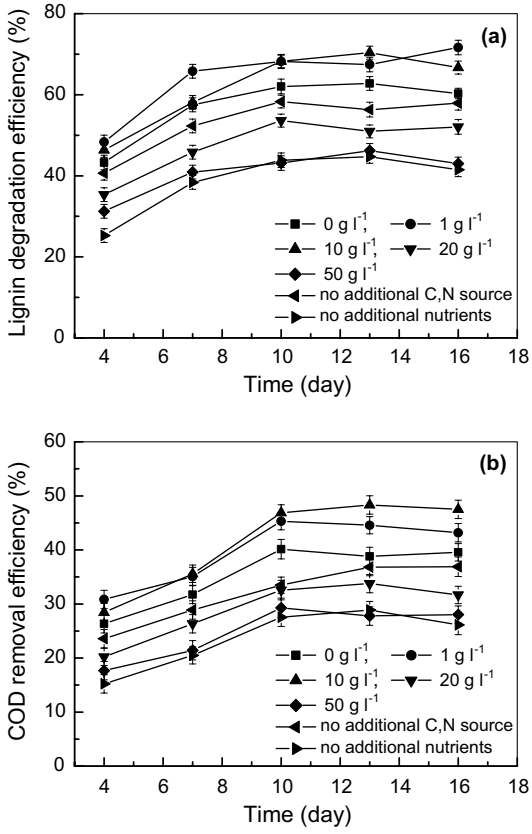

Fig. 2. Effect of carbon source concentration on the treatment efficiency of wastewater by *P. ostreatus* with medium containing ammonium tartrate 0.2 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1} and pH 6.0. Initial lignin concentration: 0.93 g l^{-1}, COD: 4.61 g l^{-1}. (a) Lignin degradation efficiency; (b) COD removal efficiency.

concentration of 10 g l^{-1} the corresponding lignin removal efficiencies were 58% and 68%. It suggests that the lignin degradation rate at 1 g l^{-1} glucose was faster than that at 10 g l^{-1} glucose. A further increase in carbon source concentration resulted in a decreased lignin removal efficiency. At higher carbon source concentrations, i.e. 20 g l^{-1} and 50 g l^{-1}, the lignin removal efficiency declined rapidly.

Fig. 2a and b show that the addition of trace elements substantially enhanced the lignin degradation. With the absence of carbon, low lignin removal efficiencies were observed, even when high levels of nitrogen and trace elements were present in the wastewater media. This demonstrates that it was essential to dose adequate carbon source, but that presence of excessive carbon source did not enhance the degradation, or even inhibited the lignin degradation.

As illustrated in Fig. 2b, the COD removal showed a similar trend to the lignin degradation. The COD removal efficiency increased by only 3% as the glucose concentration increased from 1 g l^{-1} to 10 g l^{-1}. However, a significant decrease was observed for the media with 20 g l^{-1} and 50 g l^{-1} of glucose. In the latter case, only 25%–35% of COD was removed. The lowest removal of COD was observed with no addition of nutrients. These results show that, under the conditions tested in this study, the optimum glucose dosage was 1 g l^{-1}, and that excessive glucose was not necessary. However, it also suggests that *P. ostreatus* could utilize the carbon-nitrogen sources and other nourishments to degrade lignin and to reduce COD.

The white-rot fungi are well known for their remarkable enzymatic complex capable of degrading lignin in wood (Tien and Kirk, 1983). Lignin peroxidase, Mn peroxidase and laccase are common to many white-rot basidiomycetes (Alessandro et al., 1999, 2000). Addition of glucose promoted the growth of white-rot fungi, as they needed an additional readily metabolizable carbon source for growth. Thus, the white-rot fungi would preferentially metabolize the added glucose, rather than degrading lignin. On one hand, at a low concentration of 1 g l^{-1}, glucose in the medium might promote the secretion of ligninolytic enzymes and enhance the fast biodegradation of lignin. On the other hand, as reported by Elisa et al. (1991), the removal of color from kraft mill wastewater was significant only at an appropriate range of carbon source concentration. Excessive carbon source resulted in a decline in enzyme activity of lignin peroxidase, Mn peroxidase, laccase and β-glucosidase, and therefore reduced the removal efficiency of COD from effluent.

3.3. Effect of nitrogen source concentration

In this test, ammonium tartrate was used as the nitrogen source and *P. ostreatus* was employed as the inoculum. The pH of wastewater was kept at 6.0. As shown in

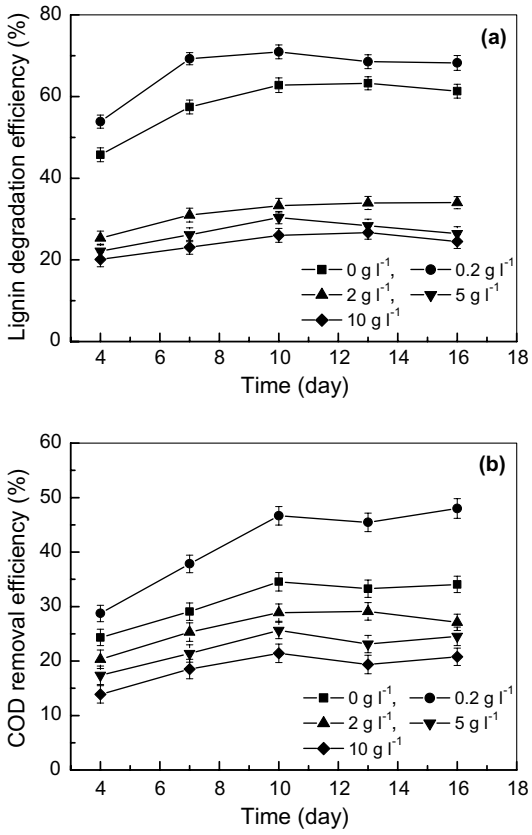

Fig. 3. Effect of nitrogen source concentration on the treatment efficiency of wastewater by *P. ostreatus* with medium containing glucose 1 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1} and pH 6.0. Initial lignin concentration: 0.93 g l^{-1}, initial COD: 4.60 g l^{-1}. (a) Lignin degradation efficiency; (b) COD removal efficiency.

Fig. 3a and b, the influence of nitrogen concentration on the removal of lignin and COD was significant. The highest removal efficiencies, 71% for lignin and 48% for COD, were achieved with 0.2 g l^{-1} ammonium tartrate. The removal efficiencies for lignin and COD decreased as ammonium tartrate concentration increased to 2 g l^{-1}, 5 g l^{-1} and 10 g l^{-1}. The lowest removal efficiencies for lignin and COD were 27% and 21%, respectively, at 10 g l^{-1} ammonium tartrate. These results suggest that a low concentration of nitrogen did enhance the lignin degradation and COD removal by *P. ostreatus*, while a high concentration of nitrogen probably inhibited the production of lignin-degradation enzymes and resulted in a decrease in lignin degradation. A previous study has showed that a low level of nitrogen could promote the degradation of lignin by *P. chrysosporium*, while a high level of nitrogen could inhibit the degradation (Kirk et al., 1978).

3.4. Effect of pH

To find out the optimum pH of fungi for lignin biodegradation and COD removal, three white-rot fungi,

P. chrysosporium, *P. ostreatus* and S22, which had better performances than the two others, were selected. The effects of pH on the removal of lignin and COD from wastewater medium by the three fungi are illustrated in Fig. 4a and b, the data in the figures being the average values from day 8 to day 12 of incubation. The highest removal efficiencies of lignin and COD were 78% at pH 9.0 and 65% at pH 8.0, respectively for *P. chrysosporium*, while the corresponding values were 84% and 69%, respectively, at pH 10.0 for S22. This indicates that the white-rot fungi tested were able to degrade lignin and to remove COD over a wide pH range, and that alkaline conditions were favorable. All these strains could form biofilm even under alkaline conditions. The optimum pH range for the different strains varied, pH 8.0–9.0 for *P. chrysosporium*, and pH 9.0–11.0 for *P. ostreatus* and S22. Since the pH of black liquor is usually higher more than 11.0, this study demonstrated the potential application of white-rot fungi for the treatment of alkaline pulp and paper wastes.

Haddadin et al. (2002) reported the bio-degradation of lignin in olive pomace by five species of basidiomycete, and studied the effect of pH on lignin degradation and enzyme production. They found that a pH of 4.5 was the optimum for enzyme production and lignin degradation by *P. chrysosporium*, while a pH of 5.0 was more appropriate for *Oxysporus* sp. and *Schizophyllum commune*. Kaal et al. (1995) demonstrated that the ligninolytic enzyme production in the wild type *P. ostreatus* was not suppressed by the unusually high pH of 6.5. Lignin mineralization by both *P. chrysosporium*, *L. edodes* and *Pleurotus sajor-caju* was shown to occur at pH 6.5 (Boyle et al., 1992). Knaap et al. (1997) observed that initial pH affected decolourization of Orange II, and that the optimal pH was in the range 5.5–6.3. These early studies emphasized the necessity of pH control in lignin degradation. However, no published data on lignin degradation at higher pH (>6.5) by white-rot fungi are available. Thus, no comparison of the data from the present work with previous results could be made.

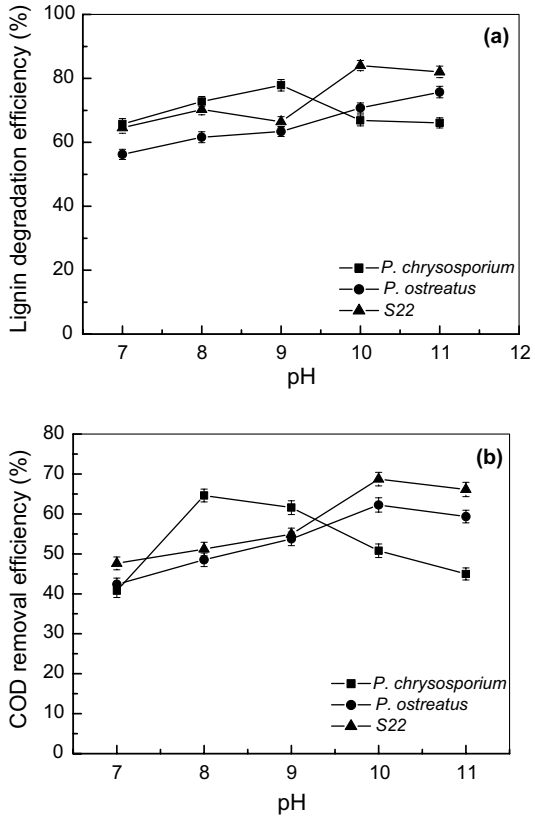

Fig. 4. Effect of pH value on the treatment efficiency of wastewater by three white-rot fungi with medium containing glucose 1 g l^{-1}, ammonium tartrate 0.2 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}. Initial lignin concentration: 0.94 g l^{-1}, initial COD: 4.62 g l^{-1}. (a) Lignin degradation efficiency; (b) COD removal efficiency.

Although lignin peroxidase, Mn peroxidase and laccase secreted by white-rot fungi are well known for their remarkable lignin degradation (Tien and Kirk, 1983; Alessandro et al., 1999, 2000), the ligninolytic enzyme systems were different for the three white-rot fungi studied. Moreover, it should be noticed that both the type and products of these enzymes changed under the alkaline conditions. This may have been due to the fact that the fungi used in this study became adapted to the alkaline conditions of black liquor. However, this warrants a further investigation.

3.5. Evaluation of the five fungi

Lignin is the main pollutant in black liquor. Because inter-unit bonds in lignin are not hydrolysable, it could not be readily degraded biologically. White-rot fungi are the sole microorganisms secreting the ligninolytic enzymes Lip, MnP and laccase, and are able to degrade lignin thoroughly. Previous work on screening lignin-degrading fungi had shown that the enzymes involved in lignin biodegradation were different among the above five white-rot fungi (Wu et al., 2002). For instance, *P. chrysosporium* produces LiP and MnP only, but no laccase.

All the five white-rot fungi tested in this work had potential applications for the treatment of black liquor, as they degraded lignin effectively. Among the five white-rot fungi, *P. ostreatus* had the highest lignin degradation efficiency. The secretion of ligninolytic enzymes, including LiP and MnP, by white-rot fungi is an idiophasic process associated with the cessation of primary growth in response to nitrogen and carbon starvation of the fungus. This association results in uncoupling of mycelial growth and enzyme production. Relatively high enzyme activities can be obtained only when carbon and nitrogen sources are limited. The experimental results reveal that a low glucose concentration of 1 g l^{-1} was beneficial

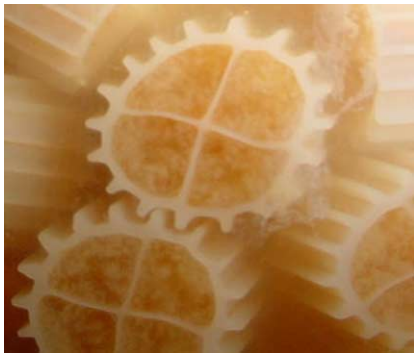

Fig. 5. Attached growth of *P. ostreatus* inside the medium.

to the biodegradation of lignin by *P. ostreatus*. Lower glucose and nitrogen concentrations in the medium may promote the secretion of ligninolytic enzymes and the fast biodegradation of lignin. This might be an advantage of employing *P. ostreatus* for the treatment of black liquor.

A significant difference between the present study and the other studies concerning lignin degradation by white-rot fungi was the utilization of a support medium in the present work. Porous plastic rings were found to be an effective medium for supporting attached growth of the five fungi. As illustrated in Fig. 5, *P. ostreatus* was evenly growing inside the porous medium. The result suggests that attached-growth white-rot fungi were of great potential for the treatment of black liquor.

4. Conclusions

Five white-rot fungi, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Lentinus edodes*, *Trametes versicolor* and S22, grown on a porous plastic medium, were used to degrade lignin in black liquor from a pulp and paper mill. Over 71% of lignin and 48% of COD were removed from the wastewater medium. Concentrations of carbon, nitrogen and trace elements in wastewater, and pH had significant effects on the growth of fungi, the degradation of lignin and the removal of COD. The addition of 1 g l^{-1} glucose and 0.2 g l^{-1} ammonium tartrate was beneficial for the lignin degradation by white-rot fungi. Three white-rot fungi, *P. chrysosporium*, *P. ostreatus* and S22, showed high capacity for lignin degradation at pH 8.0–11.0, suggesting that the white-rot fungi were able to grow and degrade lignin even under strong alkaline conditions.

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