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Degradation of chlorinated lignin compounds in a bleach plant effluent by the white-rot fungus *Trametes versicolor*

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Summary. Chlorinated lignin derivatives in a combined bleach plant effluent from sulphite pulping were degraded by several white-rot fungi among which *Trametes versicolor* (*Coriolus versicolor*) strains were the most efficient. With glucose as co-substrate, about 90% colour reduction was achieved within 3 days. Simultaneously, the concentration of chloro-organic compounds measured as adsorbable organic halogens decreased by about 45%. As shown by gel chromatography, the high-molecular-weight fraction in the effluent was completely depolymerized while over 50% of total aromatic compounds were degraded. The presence of a co-substrate was necessary for all these activities of the fungus. The residue obtained after degradation was extremely recalcitrant and not further degradable.

Introduction

Pulp-mill effluents from bleaching procedures are dark brown due to their content of chromophoric, polymeric lignin derivatives. Moreover, chlorinated organic components have toxic and mutagenic properties (Rogers 1973; Ander et al. 1977; Eriksson et al. 1979; Kringstad et al. 1981) and may accumulate in aquatic organisms (Landner 1979). The fate of such chlorolignins in ecosystems has to be studied in more detail because aquatic pollution by discharging large amounts of these effluents into streams and oceans may result in serious environmental problems.

Whereas conventional methods of waste-water treatment such as activated sludge or aerated lagoons are rather ineffective for the biodegradation of waste-water lignins, wood-degrading terrestrial fungi seem to be the most promising organisms for this particular purpose. Most studies in this field have been done with kraft bleach plant effluents and efficient decolorization has already been obtained with various white-rot species (Marton et al. 1969; Fukuzumi et al. 1977). Degradation has been particularly studied with *Phanerochaete chrysosporium* (Eaton et al. 1980; Sundman et al. 1981) and optimization of colour removal was achieved by immobilized mycelia in the MyCoR process (Eaton et al. 1982). Besides decolorization, some dechlorination of chloro-organic compounds has also been observed (Huynh et al. 1985). Addition of a co-substrate proved to be essential for effective degradation in all cases. Moreover, decolorization by *P. chrysosporium* strongly depended upon the strain used (Augusta et al. 1986). The origin of the bleach plant effluent is also important for decolorization efficiency and generally effluents from sulphite pulping seem to be more recalcitrant than those from sulphate pulping (Farcher et al. 1985).

One of the white-rot fungi known to decolorize kraft mill effluents from sulphate pulping is *Trametes versicolor*. Efficient colour removal from such effluents was obtained with calcium-alginate-immobilized mycelium in batch cultures (Livernoche et al. 1981, 1983) and in a continuous process (Royer et al. 1983). Interestingly, this fungus is also able to brighten hardwood kraft pulp (Kirkpatrick et al. 1989).

In a previous paper we described for the first time the degradation of bleach plant chlorolignins from sulphite pulping by *T. versicolor* (Bergbauer et al. 1989). Here we report the culture conditions and their optimization for *T. versicolor* strains regarding decolorization, adsorbable organic halogen (AOX) reduction, depolymerization and degradation of chlorolignins derived from sulphite pulping.

Materials and methods

Organisms. All fungi used in this study are listed in Table 1. *T. versicolor* (*Coriolus versicolor*) CBS 297.33 and *Schizophyllum commune* CBS 266.60 were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. All other strains are own isolates. Stock cultures of the fungi were stored on malt agar at 10° C and periodically subcultured.

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Table 1. Colour reduction from a combined bleach plant effluent by various white-rot fungi after 6 days incubation under standard conditions

Fungal strain	Colour reduction (%)
Trametes versicolor T	74
T. versicolor GA	74
T. versicolor GI	72
T. versicolor GB	72
T. versicolor EX	72
T. versicolor CBS 297.33	71
T. versicolor G	70
T. versicolor G II	70
T. versicolor B	67
Pleurotus ostreatus	59
T. suaveolens	55
Phlebia radiata	54
Xylaria hypoxylon	45
Bjerkandera adusta	12
Merulius tremellosus	8
Heterobasidion annosum	4
Daedaleopsis confragosa	0
Schizophyllum commune CBS 266.60	0
S. commune K	0
S. commune BBA	0

Bleach plant effluent. Samples of a combined bleach plant effluent, pH 3.4, were obtained from a commercial sulphite pulp mill. The dark brown and turbid effluent was stored at 4° C and used within 3 months. Before use the effluent was filtered through a 0.2-µm pore size filter to remove suspended particles. After filtration the effluent was still dark brown but clear and contained about 760 colour units/l.

Culture conditions. If not otherwise indicated the following standard conditions were used: bleach plant effluent was supplemented with glucose (0.8%, w/v) and basal medium including minerals and vitamins according to Kirk et al. (1978) modified with $(NH_4)_2SO_4$ (12 mM) instead of ammonium tartrate as the N-source, buffered with KH_2PO_4/K_2HPO_4 to pH 5.5 and sterilized by filtration through a 0.2-µm pore size filter (ME 24, Schleicher and Schuell, Dassel, FRG). Inoculation was done with mycelia precultured in malt medium (20 g/l, pH 5.5) after washing and disintegration of the mycelial pellets in a Waring blender. Cultures were incubated in 500-ml erlenmeyer flasks with 150 ml medium for 6 days on a rotary shaker (135 rpm) at 24° C.

For studying the influence of available nitrogen, $(NH_4)_2SO_4$ was added at either 1.2, 12 or 60 mM.

The influence of co-substrate on decolorization was tested without co-substrate and with either glucose, maltose, mannose, sucrose, xylose, potassium acetate, dipotassium oxalate, ethanol or glycerol, each at a concentration of 2% (w/v). Additionally, glucose was tested at a concentration of 0.1% (w/v).

For optimization of degradation rates a New Brunswick Scientific (Edison, NJ, USA) laboratory fermentor BioFlo II was used with the same medium but without addition of buffer. The fermentor vessel was sterilized by autoclaving and subsequently filled with filter-sterilized medium. Regulation of pH at a constant value of 5.0 was done with 0.5 M NaOH and 0.5 M HCl. Inoculation with mycelial pellets (1.3 g dry weight/l) was carried out as described above but incubation was performed with an aeration of 1 vvm at 150 rpm and 28° C for at least 6 days.

Determinations of colour units. The pH of each aliquot was measured and adjusted to 7.6 with 2 M NaOH. Colour was determined with a Beckman (Waldwick, NJ, USA) spectrophotometer 35 at 465 nm. According to Eaton et al. (1980) one colour unit is the amount of coloured material in 1.0 ml giving an absorbance of 1.0 at 465 nm and pH 7.6 by a path length of 1.0 cm.

Adsorbable organic halogens (AOX). The amount of chloro-organic compounds was determined as AOX according to the German standard method (N.N. 1985).

Molecular size distribution. Changes in the molecular size distribution were monitored by gel permentation chromatography with a Sephadex G-50 column, 2.5 cm in diameter and 100 cm in length, using 0.75 M NaCl, pH 7.0, as solvent. Six millilitres of each sample were applied to the top of the column, elution was performed at 26 ml/h and fractions of 4.5 ml were collected. Chromatograms were obtained by continuously monitoring the extinction at 280 nm with an LKB (Uppsala, Sweden) 2238 Uvicord S2 spectrophotometer.

Determination of aromatic compounds. Quantitative determination of aromatic compounds in the bleach plant effluent was carried out spectrophotometrically at 280 nm with a Beckman spectrophotometer 35. Additionally, degradation rates were determined planimetrically from the gel chromatographic curves using a planimeter (Haff, Pfronten, FRG; 315 E). Both methods gave essentially the same values.

Results

Twenty strains of white-rot fungi, including nine different isolates of *T. versicolor* and three strains of *S. commune* were tested under standard conditions for their ability to eliminate chromophoric material from a combined bleach plant effluent (Table 1). Neither the *S. commune* strains nor *Daedaleopsis confragosa* showed any bleaching activity. *Heterobasidion annosum*, *Merulius tremellosus* and *Bjerkandera adusta* merely produced a slight decolorization of the effluent between 4 and 12% within 6 days. Higher degrees of colour reduction could be achieved with the ascomycete *Xylaria hypoxylon* (45%), with *Phlebia radiata* (54%), *T. suaveolens* (55%), and *Pleurotus ostreatus* (59%). However, the most efficient decolorization (67–74%) was obtained with the nine *T. versicolor* strains. Two strains, GA and T eliminated 74% of the chromophores. The latter strain was selected to further optimize decolorization and to study the degradation in more detail.

To elucidate the influence of addtional nitrogen (total amount of nitrogen in the filtered bleach plant effluent was less than 0.0003%) for the decolorization, strain T was incubated with 0.8% (w/v) glucose combined with three different concentrations of ammonium sulphate (Table 2). The C/N ratios indicated merely refer to the final values established by the additives. Carbon sources contributed by the lignin compounds were not taken into account because of their unknown

Table 2. Decolorization of bleach plant effluent by *T. versicolor* strain T under standard conditions but using three different nitrogen concentrations

$(NH_4)_2SO_4$ (mM)	C/N ratio	Colour reduction (%)
1.2	70.0	78
12.0	7.0	81
60.0	1.4	79

C/N values refer merely to glucose and individual nitrogen concentrations added

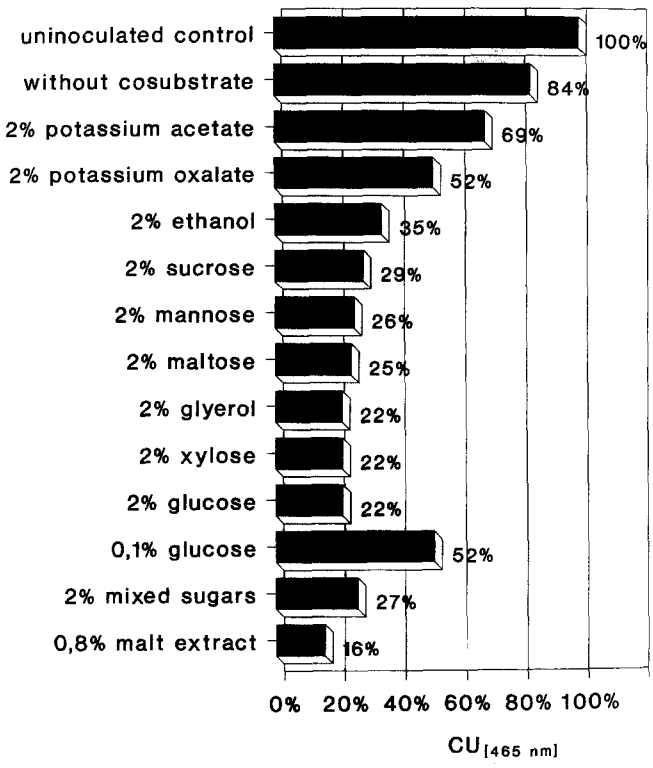

Fig. 1. Influence of various co-substrates on colour reduction in bleach plant effluent by *Trametes versicolor*. Standard conditions were used except for the variation in co-substrates. Sucrose, mannose, maltose, xylose and glucose each at a concentration of 0.4% (w/v) were used as mixed sugars (2%). CU, colour units

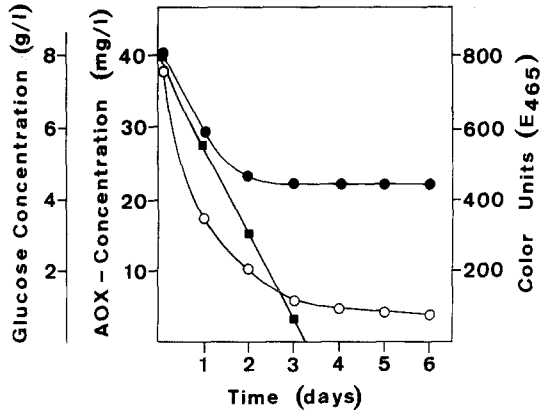

Fig. 2. Reduction in adsorbable organic halogen (AOX) concentration and colour of bleach plant effluent by *T. versicolor* within 6 days of cultivation in a laboratory fermentor: ■, glucose consumption; ●, AOX concentration; ○, colour units

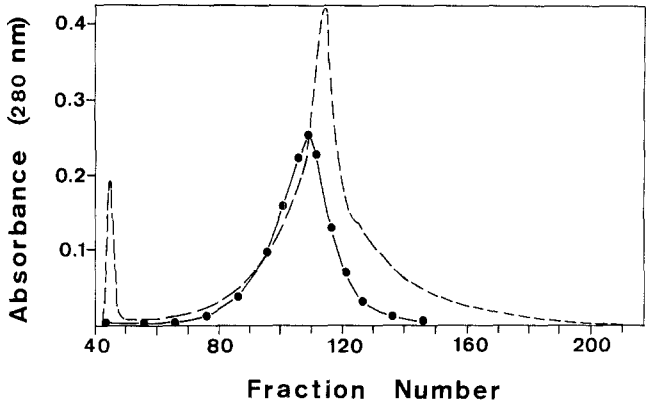

Fig. 3. Depolymerization of high-molecular-weight fractions and degradation of bleach-plant effluent compounds [E 280] by *T. versicolor* after 2 days of cultivation in a laboratory fermentor. Column material: Sephadex G-50 with 0.75 M NaCl, pH 7.0, as solvent: ---, uninoculated control; —●—, *T. versicolor* with glucose

availability to *T. versicolor*. As can be seen neither N-limiting conditions (1.2 mM ammonium sulphate, C/N = 70) nor N-supply in excess (60 mM ammonium sulphate, C/N = 1.4) had any significant effect on the decolorization rate under the conditions tested here.

In contrast, Fig. 1 shows the dependence of bleaching activities upon the kind and the concentration of added co-substrates in the presence of 12 mM ammonium sulphate. Even without any additional co-substrate, a minor colour reduction of 16% took place. With potassium acetate 31% and with dipotassium oxalate 48% colour reduction was measured, but the highest decolorization degrees in the range of 80% within 6 days were obtained with either xylose, glucose or glycerol. It is interesting to note that lowering the glucose concentration from 2% to 0.1% (w/v) reduced decolorization from 78% to 48%.

In addition to these defined compounds, malt extract and molasses as complex co-substrates were also tested, resulting in about 80% colour reduction in both cases. This degree of decolorization corresponds to that obtained by adding 2% (w/v) of either glucose, xylose or glycerol.

A further increase in decolorization rate was achieved by incubating *T. versicolor* T with bleach plant effluent in a 3-l laboratory fermentor with 0.8% glucose and 12 mM ammonium sulphate but with a constant pH value of 5.0 and under optimal aeration. As shown in Fig. 2 a reduction of the colour units (CU) from 760 CU/l initially to 87 CU/l (88% reduction) was obtained within 3 days. Compared with the 80% reduction within 6 days in flask cultures, nearly the same decolorization degree was obtained in half the time. Simultaneously, the concentration of AOX dropped from 40.0 mg/l initially to 21.9 mg/l (45% reduction) within 2 days. With malt extract (8 g/l) instead of glucose, reduction of CU as well as AOX values were nearly the same as above.

Gel chromatography revealed that a significant depolymerization and degradation of aromatic lignin compounds took place during incubation. After 3 days the added glucose was depleted and degradation ceased. By this time, the high molecular weight fraction peak had disappeared and over 50% of the aromatic compounds, mostly from the low molecular weight fraction, were also eliminated (Fig. 3). From experiments using the isolated high molecular fraction ($\geqslant$30000 Da) we know that sequential depolymerization does not necessarily lead to complete degradation of these aromatic compounds (data not shown).

Once degradation of lignin derivatives by *T. versicolor* had reached a certain level, neither additional co-substrate nor inoculation of the culture filtrate with fresh mycelium resulted in any further degradation. Therefore, the remaining recalcitrant portion of the effluent was incubated with a bacterial consortium from activated sludge, but even after 6 days no significant changes could be observed. Even subsequent incubation with a mixed culture of *Fusarium eumartii*, *Exophiala jeanselmei* and *Rhodotorula glutinis*, known for their ability to degrade lignin monomers, did not alter the final values obtained with *T. versicolor* (results not shown).

Discussion

Within the heterogeneous group of white-rot fungi, strains of *Phanerochaete chrysosporium* are usually considered the most suitable for efficient degradation of the recalcitrant chromophoric material in bleach plant effluents. For *P. chrysosporium*, two bleaching methods have been developed. In the MyCoR process, immobilization of the fungus was performed on filter paper (Eaton et al. 1982) whereas in the MYCOPOR process polyurethane was used for immobilization (Messner et al. 1988). With the latter technique, the authors obtained maximal values for colour reduction in bleach plant effluents from sulphite pulping of 87% and for AOX reduction of 80% within 48 h.

The results obtained with our *T. versicolor* strain T, 88% colour reduction and 45% AOX reduction within 48 h in a laboratory fermentor, are remarkable insofar as these values were achieved without any further biotechnological optimization such as immobilization of the fungus. Probably also in our system the time needed to reach the maximal degradation degree may be further shortened by immobilization. However, our results also suggest that the bleach plant effluent contains a very recalcitrant fraction of aromatic compounds that seem to be degradable neither by *T. versicolor* nor by other fungi and bacteria.

The only effect attainable by immobilizing *T. versicolor* would therefore be to (i) shorten the time in which the degradable compounds were eliminated and (ii) extend the active life span of the hyphae and as a consequence of this to establish a fungal system for repeated treatment of effluent by the same culture. Immobilization of *T. versicolor* in calcium alginate has already been described, but this method would be too expensive for biotechnological waste-water treatment (Livernoche et al. 1983). Preliminary experiments with polyurethane immobilization of *T. versicolor* in our laboratory showed excellent spreading of the hyphae within the rigid foams as well as strong decolorization of the bleach plant effluent.

For all the biodegradation activities mentioned above a suitable co-substrate was essential. The weak decolorization observed without added co-substrate may be due to endogeneous storage material derived from preculture in malt extract.

In contrast to *P. chrysosporium*, nitrogen limitation does not stimulate decolorization by *T. versicolor*. Since degradation of lignin derivates ceased when the fungus entered the stationary phase, secondary metabolism is not required, as also suggested by other authors (Archibald et al. 1989) for this fungal species. Interestingly, degradation of synthetic lignin by *Coriolus versicolor* was shown to be nitrogen sensitive (Leatham and Kirk 1983). Besides this apparent discrepancy it is still unclear if any of the classical lignin-degrading enzymes such as ligninase or Mn-dependent peroxidase is at all involved in the degradation of lignin derivatives in bleach plant effluents. Experiments regarding the role of extracellular enzymes, in particular laccases, in this degradation process are in progress in our laboratory.

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