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Degradation of Low and High Molecular Weight Fractions of Softwood Bleachery Effluents by *Penicillium camemberti* in Up-flow Column Reactor

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Pulp and Paper industry wastewaters and especially spent liquors from chlorine pulp bleaching have been considered for several years to be a real world wide environmental problem. The treatment of the effluents from pulp mills has been extensively studied because of the large quantities of wastewaters produced and for their toxicity and ecotoxicity. Lignin degradation products dominate the spent liquors from chlorination and alkali extraction. Spent chlorination and alkali extraction liquors from the bleaching of softwood kraft pulp are mildly toxic to fish and other aquatic organisms (Kringstad and Lindström 1984). The chlorinated compounds found in effluents are reported to be often resistant to chemical and biological degradation. Many of these compounds are toxic to aquatic organisms and several of them are genotoxic (Rosenberg et al. 1991).

It was reported that in spent chlorination liquor, about 70% of the organically bound chlorine is present as high molecular weight material (MW >1000); whereas in alkali extraction liquor, about 95 % of the organically bound chlorine belongs to this class. High molecular weight materials in spent chlorination and alkali extraction liquors are probably biologically inactive, because they cannot penetrate the cell membranes of living organisms. However, such materials are still having environmental significance as they carry chromorphoric structures that to impart light-absorbing qualities to receiving waters. About 30 % of the organically bound chlorine in spent chlorination liquor and approximately 5 % in alkali extraction liquor is of low molecular weight (MW<1000). The low molecular weight chlorolignins are already known to be toxic, mutagenic and bioaccumulative, because of their ability to penetrate the cell membranes (Kringstad and Lindström 1984; Rosenberg et al. 1991).

Pellinen and Joyce (1988) reported that the low molecular weight fractions contained more dissolved organic matter that is more efficiently removed during biological treatment. Biological treatment processes are not able to remove color and chlorinated high molecular weight organics from effluents because the organics are inaccessible to bacteria. Fungi release a family of extracellular enzymes, which can degrade high molecular weight organic compounds to the point that it can be completely mineralized or further degraded by bacteria.

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It was reported by Taşeli and Gökçay (2005) that chlorinated organic compounds like PCP, 2-chlorophenol and trichloroacetic acid those are known to be rich in pulp and paper plant bleachery effluents were effectively degraded by *Penicillium camemberti*. Treatment of pulp and paper plant bleachery effluents (chlorinated organic compounds) by an up-flow column reactor, design of a column reactor and potential field application of the fungus were published before (Taşeli et al. 2004).

The aims of this study was to examine (1) the best solid matrix to be used to fill the up-flow column reactor in which *Penicillium camemberti* can be immobilized on and (2) the ability *Penicillium camemberti* to degrade low and high MW fractions of softwood pulping and bleaching effluents in an up-flow column reactor.

MATERIALS AND METHODS

Wastewater samples obtained from Turkish State Paper Industries' (SEKA) Dalaman Pulping and Paper Plant were used for the continuous column experiments. Dalaman pulping plant uses mainly pine softwood for raw material. Kraft process is employed for pulp production and cellulose is cooked in a vertical Camyr pressurized cooker. A six stage bleachery process is applied to the cooked pulp in the following sequence: Chlorination (C), Alkali extraction with caustic soda (E), Hypochlorite (H), Chlorine dioxide (D), Alkali extraction (E) and Chlorine dioxide (D) stages. The abbreviation CEHDED is used in this paper to mean bleachery effluents.

The *Penicillium camemberti* isolate used in this study was isolated from chlorination-stage acidic effluents of SEKA Pulp and Paper Plant in Kastamonu in Turkey. The isolated fungus was identified through elaborate biochemical tests (Pitt 1993). For determination of biodegradation, the basal medium (0.2 g/l of acetate, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.1 g/l $CaCl_2$, 0.12 g/l NH_4Cl and 0.001g/l thiamine) was supplemented with chlorinated pulping effluents. The pH was adjusted to 5 and temperature to 25±2°C since in an Author's earlier study, the optimum temperature and pH was found as 25±2 °C and 5.0, respectively (Gökçay and Taşeli 1997). Batch culturing was carried out in 500 ml conical flasks that were inoculated with 10 ml of a spore suspension (optical density of 0.5 at 650 nm) and incubated on a rotary shaker at 80 rpm.

Adsorbable organic halogens (AOX) analyses were carried out according to German DIN 38409 Norm. The soluble organics were first adsorbed onto pure activated carbon particles and then they were filtered off on polycarbonate filters, washed with a nitrate solution and combusted in the furnace of the Euroglas 500 AOX analyzer. The chloride release was detected and recorded by the instrument as mg/l AOX.

Gas chromatography analyses were carried out by Perkin Elmer Autosystem 1020 Plus Gas Chromatograph. Firstly, gas chromatographer was calibrated with

standard mix solution including target compounds. The calibration procedure was repeated prior to samples every 5 samples. Secondly, effluent samples were first pre-conditioned with methanol and then were passed through C18 solid phase extraction columns. Organics retained on the C18 column were eluted with freshly distilled chloroform. The collected chloroform phase was dried by passing through anhydrous Na_2SO_4 and further concentrated down to 0.1 ml in a micro Kuderna Danish concentrator. The concentrated samples were then injected to and analyzed using the gas chromatographer with electron capture detector and CP Sil-5 capillary column.

Total organic carbon (TOC) content of the effluents was determined using the total organic carbon analyzer, model 1555B, Ionics. It was calibrated upon start up at laboratory and the calibration was checked at least once a week using a standard solution of known concentration. In experiments, a wastewater sample of either 20 µl, 100 µl or 200 µl (volume depends on the range) was injected into a reaction chamber, packed with a catalyst and held at a fixed temperature. Samples were analyzed for total carbon (TC) and total inorganic carbon (TIC). TOC content of the original sample was found by subtracting TIC from TC.

The UV scans of effluents were obtained by scanning between 200-400 nm using a Secomam UV- VIS spectrophotometer. Relative color at absorbance of 465 nm was measured using the Pharmacia Biotech Spectrophotometer.

Molecular weight (MW) distribution analyses were carried out in a 1m by 1.5cm Sephadex G-50 column, using 0.1 N LiOH-NaCl solutions as eluent. Fractions were collected and ring structures were followed with absorbance at 280 nm by using an UV spectrophotometer. The column was suitably calibrated using three reference materials with known molecular weights. After column was calibrated, the effluent was collected as 3 ml fractions from the outlet. Blue dextran was used for the determination of the void volume, V_o, which is the volume of liquid required to elute compounds that are completely excluded from the gel grains. Potassium chromate with its low molecular weight was freely accessible to the gel particles. Lysosome and poly (ethylene glycol ether) were eluted from the column in the order of decreasing molecular weight. The knowledge of effluent volume of a particular compound enables its distribution coefficient (k_d) to be calculated. Table 1 tabulates the results of calibration of Sephadex G-50.

The biological treatability experiments were conducted in a bench-scale up-flow tubular column reactor with a 6.7 cm inner diameter and 55.7 cm height. The column reactor consisted of a feed tank, a feed pump and the column itself, having an inlet, an outlet, and 4 sampling outlets. The bioreactor was operated in a continuous manner; the feed solution was either concentrate or its diluted form as per required for the experiments and was continuously supplied to the reactor on its bottom and the products were continuously withdrawn from its top. The up-flow fungal column reactor used in this study was prepared by filling a PVC column with glass wool, which was previously seeded with the isolated fungus.

RESULTS AND DISCUSSION

The *Penicillium camemberti* was tried to be immobilized on a solid matrix since immobilized systems are believed to enhance migration of toxic compounds from the environment to the adsorbent and provide a controlled microenvironment for subsequent biodegradation (Lin and Wang 1991). Three kinds of solid matrixes namely glass wool, glass pieces and polystyrene foams were tested for their ability to retain the fungal cells. Firstly, 200 ml of bleachery effluent (CEHDED) inoculated with 10 ml of fungal suspension were placed in 3 flasks, mineral salts and 0.2 g/l acetate was added to each flask. Acetate was chosen as primary carbon source for the fungus since, in an earlier study the highest biomass (dry weight/l) concentration was achieved by acetate. The other carbon sources tested were peptone, maltose, acetate, methanol, glucose and phenol (Gökçay and Taşeli 1997). Secondly, glass wool, glass pieces and polystyrene foams were added to flask no. 1, flask no.2 and flask no.3, respectively. The flaks were incubated under non-shaking conditions for 10 days with pH 5.0 and temperature 25±2°C. 10 day of incubation was chosen since it was proved by the earlier studies that 10 day was the optima for the fungus (Taşeli et al 2004; Taşeli and Gökçay 2005a). Control experiments were performed with *Penicillium camemberti* cultures that had been boiled for 10 min., which was reported to be acceptable practice for sterilization of fungi prior to supplementation with lindane (Kuritz and Wolk 1995). Thirdly, fungal biomass, AOX, TOC and color removals were determined after 10 days of incubation.

Table 2 tabulates the results of immobilization studies. The visual and microscopic examination of the solid substances at the end of 10 days of incubation indicated that only glass wool was able to successfully immobilize fungal cells. No appreciable cell mass immobilized on others. The fungus was completely immobilized on glass-wool and there was no freely suspending fungal particle in the culture fluid and supernatant was clear. The maximum fungal biomass of 1620 mg/l was measured in flask no.1. In addition, 61.93 % AOX, 51 % TOC and 59.28 % color removals were also achieved with immobilization of fungus on glass wool. There was no conspicuous immobilization of fungus on glass pieces and fungus was freely suspended in the culture liquid resulting in high turbidity. The minimum fungal biomass of 250 mg/l was measured in flask no.2. 45.2 % AOX, 51.5 % TOC and 48.19 % color removals were achieved with glass pieces. The fungus was slightly immobilized on to the polystyrene foams. The foam surfaces were not covered by slime layer. The high turbidity was observed. The fungal biomass of 310 mg/l was measured in flask no.3. 48.1 % AOX, 61.3 % TOC and 57.24 % color removals were achieved with polystyrene foams.

As a result of immobilization experiments, it was clear that glass wool was a perfect sorbent matrix for immobilization of *Penicillium camemberti* while others proved to be ineffective. Because of this reason, the up-flow fungal column reactor used in this study was prepared by filling a PVC column with glass wool, which was previously seeded with the isolated fungus.

In order to investigate the treatment efficiency of high and low MW fractions of chlorinated organics by *Penicillium camemberti*, molecular weight distribution analysis of softwood bleachery effluent before and after column treatment was performed by gel filtration method. Figure 1 shows the molecular weight distributions before (Figure 1A) and after (Figure 1B) ten days of column treatment with mineral salts and 0.2 g/l acetate as carbon source at pH 5, temperature 25±2°C and Table 3 tabulates the results. It is clear from Figure 1A that, softwood bleachery effluent consists predominantly of MW between 10,000 and 1,000 (40 %). It is followed by MW>30,000 (32 %), MW between 30,000 and 10,000 (25 %) and MW<1,000 (3%). As can be seen from Figure 1B, compounds having molecular weight higher than 30,000 g/mol, corresponding to 32 % before fungal treatment, is reduced to 19 % after fungal treatment. Molecular weight between 30,000 and 10,000 g/mol is increased from 25 % to 56 % after column treatment. In addition, compounds having molecular weight less than 1,000 g/mol are completely removed after column treatment. Fungal treatment changed the relative proportions of the chlorinated compounds in the bleachery effluent. These results are also in accord with the other researcher's observations in which biotreatment removed low MW material more efficiently than high MW material (Bryant et al. 1987; Lindström and Mohamed 1988; Fitasimons et al. 1990; Aprahamian and Stevens 1990; Jokela et al. 1993).

In the light of these findings it can be generalized that *Penicillium camemberti* treats organic chlorine compounds in the softwood bleachery effluents by completely removing low molecular weight (MW<1000) compounds while depolymerizing large molecular weight chlorolignins (MW>30,000) to oligomers.

These conclusions are also in accord with the gas chromatograms and UV scans shown in Figure 2 and Figure 3, respectively. When peaks in Figure 2 are examined, it is obvious that absorbance values at 280 nm are significantly decreased following the fungal treatment. It is also clear that there is an obvious difference between before and after fungal treatment at critical absorbance bands of 280 nm and 254 nm corresponding to phenol and aromatic ring resonances, respectively (Figure 3). The fungus effectively opens aromatic rings of the chlorolignins during treatment.

The design of an up-flow column reactor, potential field application of the fungus, the results of adsorbable organic halogen (AOX), total organic carbon (TOC) and color (465 nm) analysis were published before. It was found that, at best around, 76 % AOX, 65 % TOC and 61 % color were removed from the softwood bleachery effluents in the bioreactor in 7.3 hr of contact with no aeration and a minimal amount of carbon (0.2 g/l acetate) and mineral salts at pH 5, temperature 25±2°C (Taşeli et al. 2004).

This study showed that the continuous up-flow packed bed reactor immobilized with *Penicillium camemberti* completely removed small sized phenolics (MW<1000) implying toxicity reduction in the effluents. Moreover, medium range phenolics were also substantially reduced (MW:1000-10000) while an increase in the mezzo range (MW:10 000-30000) was noticeable.

Table 1. Calibration of sephadex G-50 column.

material	elution volume V_e (ml)	distribution coefficient k_d	molecular weight (g/mol)	extinction wavelength (nm)
potassium chromate	58	0.87	194	370
poly (ethylene glycol methyl ether)	50	0.6	5,000	275
Lysozome	38	0.2	14,300	280
blue dextran	V_o=32	0	2,000,000	625

Table 2. Results of immobilization studies after 10 days of incubation.

no	flask contents	visual examination	fungal biomass (mg/l)	AOX removal (%)	TOC removal (%)	Color removal (%)
1	200 ml CEHDED +10 ml fungal suspension + mineral salts + 0.2 g/l acetate + glass wool	Perfect immobilization	1620	61.93	51	59.28
2	200 ml CEHDED +10 ml fungal suspension + mineral salts + 0.2 g/l acetate + glass pieces	Poor immobilization	250	45.2	51.5	48.19
3	200 ml CEHDED +10 ml fungal suspension + mineral salts + 0.2 g/l acetate + polystyrene foams	Poor immobilization	310	48.1	61.3	57.24

Condition: pH 5, temperature 25±2 °C, non-shaking.

Table 3. Molecular weight (MW) distributions of bleachery effluent (CEHDED) sample before and after column treatment.

MW (g/mol)	before fungal treatment (%)	after fungal treatment (%)
>30,000	32	19
30,000-10,000	25	56
10,000-1,000	40	25
<1000	3	-

Condition: (CEHDED) + mineral salts + 0.2 g/l acetate, pH 5, 25±2 °C.

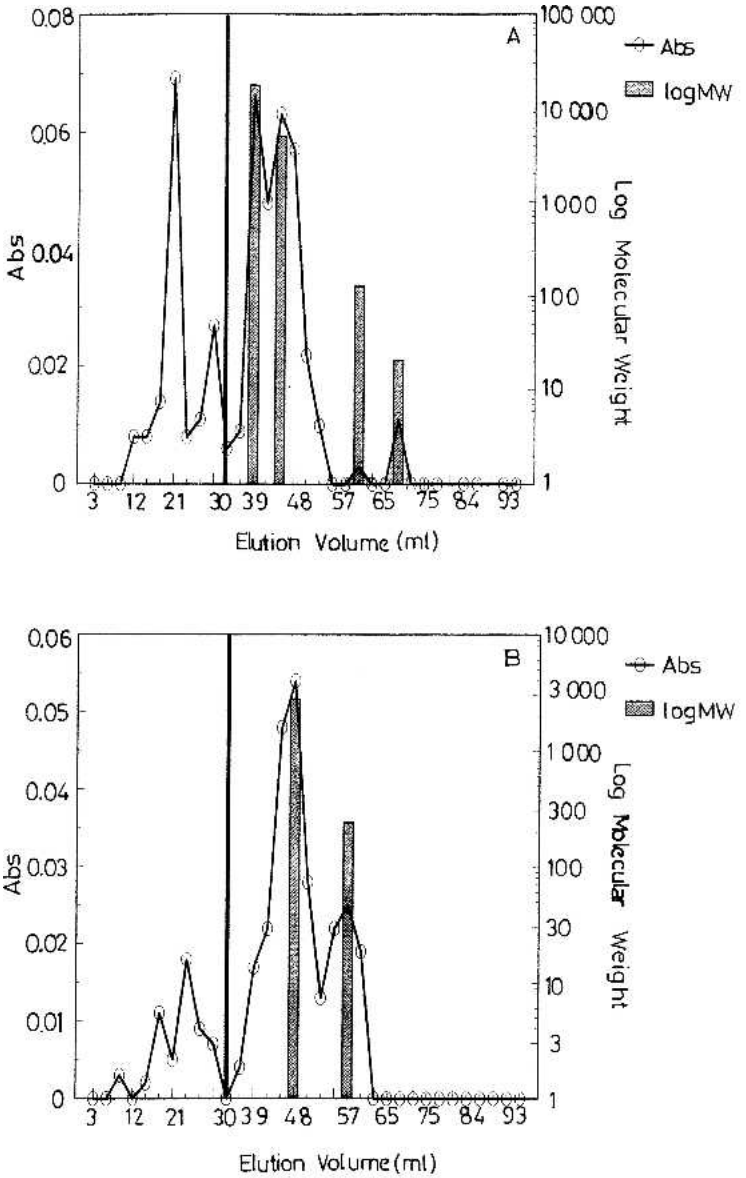

Figure 1. MW distribution of softwood bleachery effluent (CEHDED) + mineral salts + 0.2 g/l acetate before (A) and after (B) column treatment. (Note: Those with elution volume less than 32 ml have molecular weight greater than 30,000)

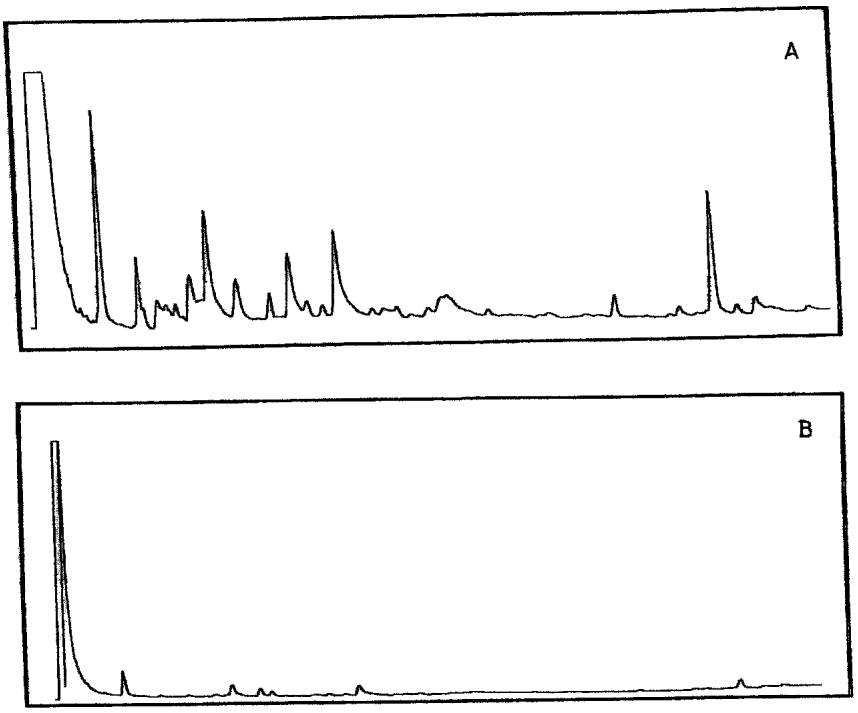

Figure 2. Gas chromatography analysis of softwood bleachery effluent (CEHDED) + mineral salts + 0.2 g/l acetate before (A) and after (B) column treatment.

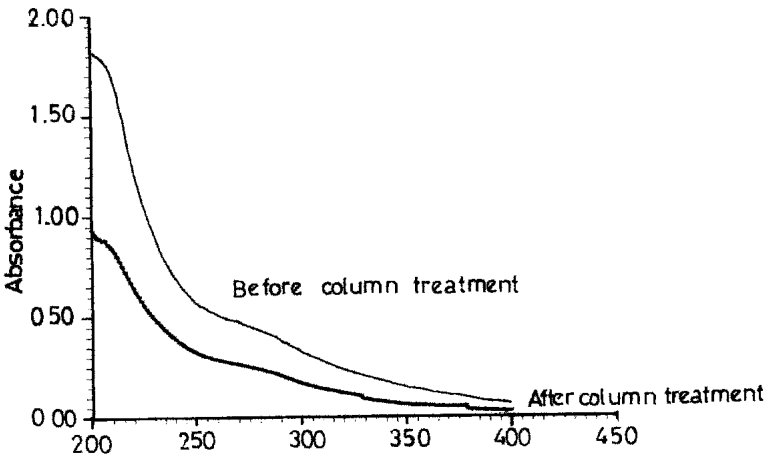

Figure 3. UV scans of softwood bleachery effluent (CEHDED) + mineral salts + 0.2 g/l acetate before and after column treatment.

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