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Monitoring Phenolic Compounds During Biological Treatment of Kraft Pulp Mill Effluent Using Bacterial Biosensors

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Kraft cellulose bleaching effluents contain toxic aromatic organic chlorinated compounds that are difficult to treat by conventional waste water treatment systems (Fleming, 1995). These include chlorinated heterocyclic and phenolic compounds derived from chemical reactions between lignin and bleaching reagents. Environmental regulation imposes strict discharge limits to such contaminants due to their potential negative effect on aquatic ecosystems (Revé and Eral, 1989). For instance, recent emission norms published by the U.S. Environmental Protection Agency target several key pollutants present in kraft pulp bleaching effluents, such as 2-chlorophenol, 2,4-dichlorophenol, 2.4.6-trichlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, and other priority pollutants (EPA, 1998). Effective implementation of such controls implies the use of accurate analytical techniques capable of detecting the presence of such key pollutants in contaminated waters and soils. Conventional analytical techniques based on spectrometry (UV, visible, IR) and chromatography (GC, TLC, HPLC) could be used to carry out such monitoring task (Martinez et al., 1996; Scheper, 1992). However, rapid low cost alternatives for routine detection of toxic pollutants would be welcome. In this respect, biosensors using genetically modified bacteria could be used as a cheaper and simpler alternative (King et al. 1991; Burlage et al. 1994; Aizawa et al. 1995; Harayama et al., 1992). Such biosensor should contain a reporter gene, e.g., β-galactosidase (*lacZ*) or luciferase (*lux*) genes, which operates under transcriptional activator control. The presence of a key contaminant stimulate the transcriptional activator, expressing the reporter gene which, in turn, could be detected by the corresponding enzymatic activity. Thus, under appropriate conditions, a direct correlation between the contaminant concentration and the enzymatic activity could be found (Willardson et al., 1998; Rasmussen et al., 1997).

Various bacterial biosensors have been described in the literature (eg. Heitzen et al., 1994; King et al., 1991; Burlage et al., 1994; Aizawa et al., 1995; Ikariyama et al., 1997; Willardson et al., 1998; Ikariyama et al. 1997; De Lorenzo et al., 1993; Applegate et al., 1998). Recently, a set of mutant bacterial strains able to detect phenolic compounds has been reported (Wise and Kuske 2000). These authors modified the phenol detection capacity of the DmpR protein by PCR mutagenesis in the DmpR sensor domain. Detection of phenolic compounds could be shown by

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the bacterial capacity to activate the transcription of *lacZ* gene, producing a readably measurable signal (ie. B-galactosidase activity).

Such bacterial system offers an attractive alternative for the detection and monitoring of organic chlorinated pollutants present in industrial effluents. Within that context, this paper reports experimental results on the capacity of three mutant DmpR strains to detect chlorinated organic compounds during biological treatment of kraft pulp bleaching effluents.

MATERIALS AND METHODS

Bacterial strains, Mutant *E.coli* AW101 DmpR B23 and B24 strains, kindly provided by Dr Kuske (Environmental Molecular Biology Group, Biosciences Division, Los Alamos National Lab, New Mexico), were used.

Synthetic effluents were prepared mixing various chlorinated phenols and non chlorinated aromatic compounds. Analytical grade chemicals assayed here included: phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, 2,4,6-trimethylphenol, 2,4,5-trimethylphenol, pentaclorophenol, 2-methyl-4,6-dinitrophenol, xylene, toluene, and benzene. All were obtained from Merck and, in each case, 25 mM stock solution in ethanol were prepared and stored for further use in sample preparations.

Kraft pulp bleaching effluents, unbleached kraft pulp (with lignin content measured as kappa number 25), from Chilean pinewood, was provided by a local mill. Oven dried unbleached pulp samples (800 g) were bleached using two bleaching sequences, one standard (C-E_{OP}-D) and other Elementary Chlorine Free, ECF (D-E_{OP}-D), where C: Cl_2, D: ClO_2, E_{OP}: NaOH extraction in the presence of O_2 and H_2O_2. Bleaching conditions for standard and ECF sequences:
-First stage: kappa factor 2.2 and 2.0; 10% and 5% consistency at 40°C for 40 min, respectively;
-Alkaline extraction (2% NaOH) in the presence of oxygen gas at 3 bar pressure, 70°C, 10% consistency for 60 min;
-Final ClO_2 treatment was carried out to obtain a bleached pulp with 90ºISO brightness.
At each stage, treated pulp was washed with a total volume of 16 dm3 demineralised water. All bleaching wastewater were collected and stored at 2°C in a refrigerator, for further analysis and treatment.

Bleaching effluents were treated by anaerobic and aerobic biological processes (Zaror, 1997; González et al., 2003). The treatment system used in this study consisted of two laboratory scale units operating in semicontinuous mode. A 5 L anaerobic contactor and a 5 L activated sludge unit were used here. The aerobic activated sludge bioreactor was aerated using and air diffuser located under the stirring blade. Both systems featured temperature and pH automated control. Biomass was adapted during 4 months operation using industrial effluents from a

local pulp mill. Total phenols were determined in raw and treated bleaching effluents by spectrophotometric standard methods (Standard Methods, 1995).

For the detection of chlorinated phenols and other aromatic compounds, DmpR mutant strains were cultivated overnight in Luria-Bertani (LB) broth in presence of tetracycline (10.5 mg/mL), a 37° C, in a stirred bath. 800 uL cultured sample featuring an absorbance in the range 0.8-1.0 at 595 nm (A595). Then, bacteria were pelletised by centrifugation and, resuspended in Luria-Bertani broth, in presence of solutions containing different concentrations of tested compounds, at 37°C during 2h, in a stirred bath. Bacterial cells were pelletised by centrifugation and refrigerated at –70°C. Similar experiments were conducted using raw and treated cellulose bleaching effluents, by resuspending 800 uL in the filtered effluent (Campos et al. 2004).

Liquid β-galactosidase assays were performed by using a modification of Miller's assay (Miller, 1992). Cell sample pellets were thawed and suspended in 800 ml of Z buffer (60 mM Na_2HPO_4 H_2O, 40 mM NaH_2PO_4 H_2O, 10 mM KCl, 1 mM $MgSO_4$ $7H_2O$). The absorbance at 595 nm of 100 uL of each cell suspension was determined in a microtiter plate by using an automated microplate reader (BIO-TEK Instruments, Inc., Winooski, Vt.). Following the addition of 15 uL of 1% (wt/vol) sodium dodecyl sulphate and 20 uL of $HCCl_3$, the remaining cell suspension was vortexed for 30 s to lyse cells, and 100 uL of each lysed sample was placed in the well of a microtiter plate. Each assay reaction was initiated with the addition of 50 uL of o-nitrophenyl-b-D-galactopyranoside (2.5 mg/ml). Reaction mixtures were incubated at 26°C, and reactions were stopped with the addition of 50 uL of 1M Na_2CO_3. Colour development for each reaction was detected by absorbance measurement at 415 nm on the microplate reader. Enzymatic activity was expressed in terms of arbitrary Miller Units (MU), corresponding to $1000xA_{415}/A_{595}$xReaction time.

Bacillus subtilis rec assay to detect the presence of any DNA damaging compounds was used here (Mazza, 1982). *Bacillus subtilis* 1652 rec(+) and *Bacillus subtilis* 1791 rec(-) strains were used. *Bacillus subtilis* strains were culture overnight in a stirred nutritive broth at 37°C. Then, 100 uL culture were inoculated in 2 mL agar Soft (two parts nutritive broth and one part of agar nutrient) at 45°C, and homogeneously distributed on agar plaques. Once agar Soft solidified, 0.5 cm diameter holes were made on each plaque and filled with 100uL effluent. Plates were maintained for 12 h at 4°C and then incubated for 24 h at 37°C. The size of resulting inhibition rings was measured, and the relative toxicity was estimated as the ratio between the sizes of rec(-) and rec(+) strains inhibition rings.

RESULTS AND DISCUSSION

Table 1 shows the DmpR mutant strains responses to chlorinated phenols and other tested organic compounds, at 100 μM concentration. Clearly, all chlorinated phenols induced enzymatic responses. However, no responses were observed in

the case of pentachlorophenol, 2-methyl-4,6-dinitrophenol, xylene, toluene and benzene. It must be said that assays were conducted al neutral pH and, given the pentachlorophenol (4.9) and 2-methyl-4,6-dinitrophenol (4.2) pK_a values (viz. 4.9 and 4.2, respectively), these molecules may find it difficult to cross the cell wall due to deprotonation of hydroxyl groups. On the other hand, toluene, xylene and benzene assays indicate the high specificity shown by the mutant DmpR strains, since those compounds are effectors of the XylR protein sensor domain, which presents 64% homology with the DmpR protein sensor domain, at aminoacid levels (Shingler and Moore, 1994).

Table 1. DmpR mutant strains responses to chlorinated phenol and other organic compounds, at 100 μM concentration.

DmpR mutant strains			DmpR mutant strains		
Compounds	**B23***	**B24**	**Compounds**	**B23**	**B24**
4-Chloro-3-Metilphenol	143	105.3	2,4,5-Trichlorophenol	36	37
2,4-Dimetiphenol	264.3	119.3	Pentachlorophenol	S/A	S/A
2,4-Dichlorophenol	259.3	126	Benzene	S/A	S/A
Phenol	268.6	315	Xylene	S/A	S/A
4-Chlorophenol	310	242.6	Toluene	S/A	S/A
3-Chlorophenol	286.6	322.3	Water MilliQ	S/A	S/A
2-Chlorophenol	310.3	301	Serum	S/A	S/A
2,4,6-Trichlorophenol	198.3	196.4	2- Methyl-4-6-dinitrophenol	S/A	S/A

*Muller Arbitrary Units (1000)(A415)/(A595)(reaction time) (Miller, 1972).

Figure 1 shows biosensor responses to 7 organochlorinated compounds. It is observed that biosensor responses increased as contaminant concentration increased. Linear regression analysis shows that DmpR mutant strains presented a linear response between 0.1 and 75 uM approximately. *B*-galactosidase activity was expressed in terms of Muller arbitrary units (Miller, 1972). All experiments were conducted in triplicate and three different cultures.

The efficiency of bleaching effluent biological treatment, under aerobic and anaerobic conditions, was assessed on the basis on DmpR mutant strains responses and total phenols determination. Figures 2a and 2b show phenolic compounds determinations using DmpR B23 y B24 strains. It is observed that a significant reduction in the effluent phenolic concentration in both treatments. Similar results were obtained by spectrometric assays. The total phenols concentration in standard bleaching effluents was much higher than that of ECF process, since Cl_2 adds to aromatic rings as opposed to ClO_2 that oxidizes and destroys the rings with lower chlorine addition.

The presence and concentration of a pollutant can be determined with greater accuracy by chemical analytical methods rather than with a bacteria biosensors. However, the biosensor reporter here provides additional unique information on bioavailability.

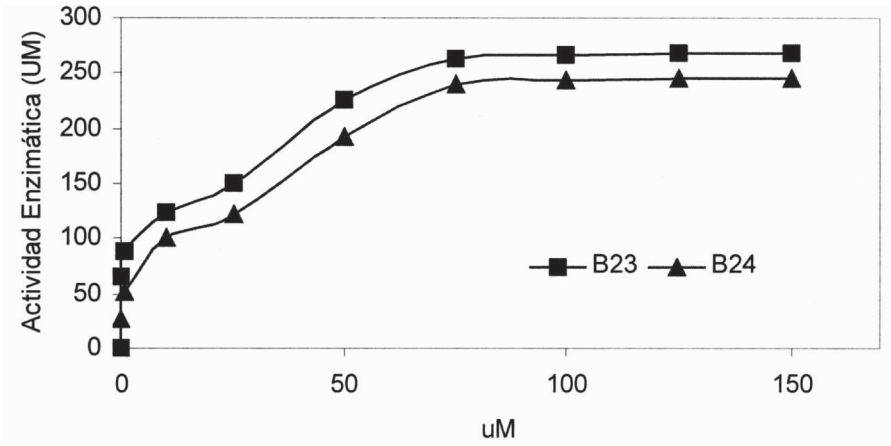

Figure 1. Response of mutant DmpR strains to chlorinated compounds mixtures.

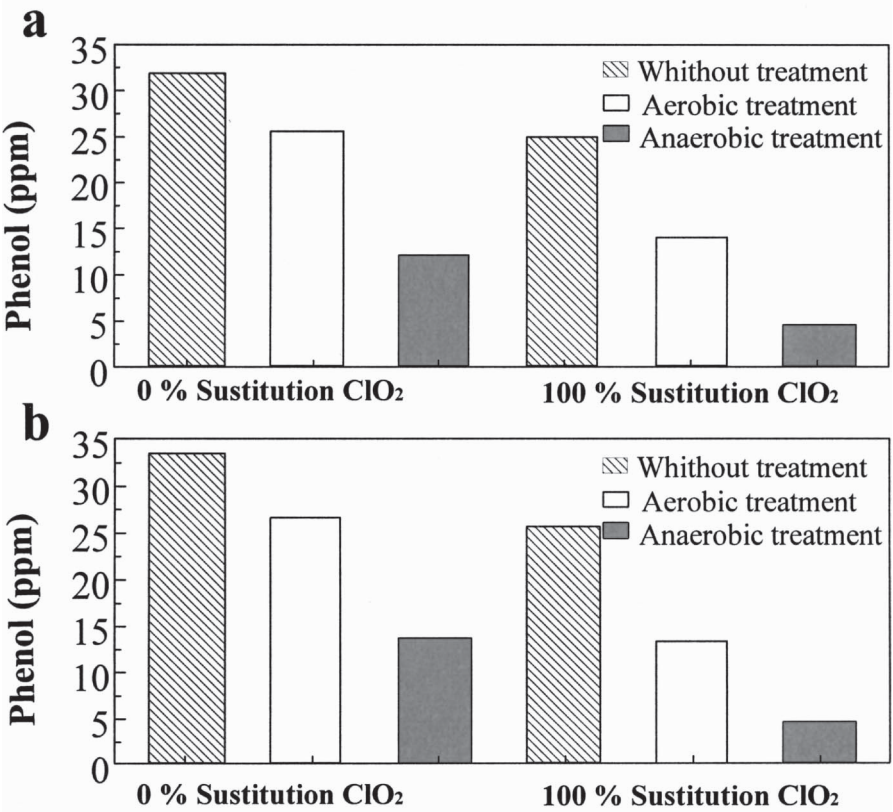

Figure 2. Phenol determination using a) DmpR B23 mutant strain b) DmpR B24 mutant strain. Pulp bleaching effluent treated by aerobic and anaerobic biological systems.

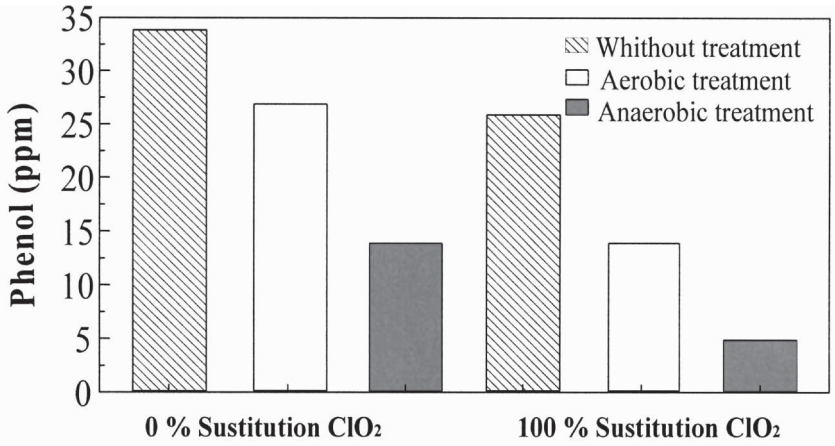

Figure 3. Total phenol determination. Pulp bleaching effluent treated by aerobic and anaerobic biological systems.

The *Bacillus subtilis* rec assay was used to determine effluents genotoxicity, and provides information on possible DNA damage induced by contaminants. Table 2 shows the relationship between inhibition rings of *B. subtillis* 1652 (Rec+) and *B. subtillis* 1791 Rec(-). Untreated effluent shows a ratio of 1.4, which is higher than the 1.0-1.2 upper limit stablished by Mazza (1982), indicating the presence of genotoxic compounds. Such ratio decreased to 1.0-1.1 after biological treatment.

Results obtained from chlorophenolic compounds mixtures. At 5 ppm chlorophenol concentration rec ratios was 1.1, whereas at 50 ppm this ratio was 1.3 indicating possible genotoxic effects at those levels.

Table 2. Effluents genotoxicity by *Bacillus subtilis r*ec assays.

Samples	Relative activity Rec (+)/Rec(-)
Untreated Effluents	1.4
Effluent from Aerobic Treatment	1.12
Effluent from Anaerobic Treatment	1.04
Chlorophenolic mixture, 5 ppm	1.1
Chlorophenolic mixture, 50 ppm	1.3

Inhibition halo was measure after incubation at 37°C for 12 h

This study showed the phenol and chlorinated phenols detection capacity of DmpR, B23 and B24, mutant strains based on the activation of *Pdmp-lacZ* fusion. Bacteria biosensors presented high sensitivity and specificity, thus could be used as an alternative to traditional chemical analytical techniques, particularly for field work and *in situ* monitoring (Heitzer 1992).

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