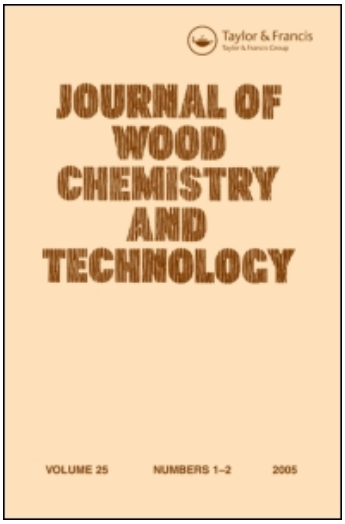

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THE FATE OF ^{14}C-LABELLED HIGH MOLECULAR WEIGHT CHLORINATED LIGNIN AND CHROMOPHORIC MATERIAL DURING MICROBIAL TREATMENT OF BLEACHED KRAFT EFFLUENT

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ABSTRACT

Bleached kraft effluent (BKE) is highly coloured due to the presence of high molecular weight, chlorinated, oxidised lignin. The dimethyl formamide-soluble portion of BKE was radiolabelled by methoxylation with ^{14}C-methyl iodide, and the resulting product (C14-BKE) was used to investigate the mechanism of colour removal by fungi and bacteria. The white-rot fungi Coriolus versicolor and Pleurotus sajor-caju decolorised the solutions, and depolymerised the C14-BKE. Both fungi produced about 16% $^{14}CO_2$ from C14-BKE in 20 days. The three bacteria tested, Bacillus cereus and two strains of Pseudomonas aeruginosa, removed colour primarily by adsorption with little depolymerisation or mineralization. The adsorbed C14-BKE could be removed from bacterial cells by washing with 0.1 M NaOH.

INTRODUCTION

Effluents from the bleach plants of kraft mills are highly coloured due mainly to the presence of high molecular weight chlorinated lignin[1] removed from the pulp largely during alkaline extraction. Although many mills have biological treatment plants, such as aerated lagoons, for reduction of biological oxygen demand and fish toxicity, the lignin is recalcitrant and remains in solution giving a highly coloured effluent. Various

white-rot fungi[2,3] and bacteria[4] have the ability to remove colour from bleached kraft effluent (BKE). However, it is difficult to tell from these reports whether colour removal is due to chemical modification of the chromophores or simply adsorption of coloured material on the biomass. Several white-rot fungi, such as Phanerochaete chrysosporium[3] and Coriolus versicolor[2] are capable of decolorising BKE on a continuous basis which involves more than simple adsorption because the adsorptive capacity of the biomass is very limited.

The mechanism of lignin and lignin model compound degradation by P. chrysosporium is becoming better understood[5]. Recently an extracellular manganese-dependent peroxidase with both colour-removing and ligninolytic activity was isolated[6]. Glenn and Gold[7] have also established a correlation between lignin biodegradation and colour removal from polymeric dyes. Colour removal from BKE by C. versicolor is effected by two independent routes[8], one involving H_2O_2 production by an intracellular oxidase and the other involving direct oxidation of chromophores by oxygen and laccase. For other microbial systems, adsorption might be an important factor. Marton et al.[9] concluded that adsorption of chromophores was an important factor in colour removal from kraft black liquor by the fungus Polyporus versicolor.

Radiolabelling has been recognised previously as a convenient method for following the metabolic fate of lignin[10]. Both in vivo labelling[11] and ^{14}C methoxylation[12] of naturally-occurring lignins have been used. For industrial lignins, Lundquist et al.[13] produced kraft and bleached kraft lignins from C14-labelled dehydrogenation polymerizate (DHP), which could be degraded and eventually mineralized by P. chrysosporium. Eriksson[14] has obtained C14-labelled BKE by pulping and bleaching in vivo labelled spruce. The BKE was mineralized by P. chrysosporium, but not by bacteria. In this paper, we describe a procedure for the simplified preparation of C14-BKE by methylation of high molecular weight BKE solids with ^{14}C methyl iodide.

Only the fungi could depolymerise and mineralize the BKE to CO_2 under our experimental conditions. The bacteria were found to adsorb the radiolabelled material.

MATERIALS AND METHODS

Organisms and Culture Conditions

Coriolus versicolor[2] and Pleurotus sajor-caju[15] were grown in shake flasks (500 mL) containing 200 mL of malt extract broth (Difco) with a 2 cm glass bead for 5 days at 30°C and 250 rpm. Pseudomonas aeruginosa, strains ATCC 31482 and SR13, and Bacillus cereus S-12 were grown for 2 days on nutrient broth as above. B. cereus and strain S-R13 were selected from a bank of 25 strains of bacteria previously screened for their capacity to remove colour, and originated from the intestinal tract of an earthworm and the activated sludge of a kraft mill, respectively.

Preparation of ^{14}C-Methylated High Molecular Weight Chlorinated Lignin

Effluent (200 mL) from the first extraction stage of the bleach plant of a west-interior Canadian kraft mill was adjusted to pH 3 and freeze dried. The resulting freeze-dried powder was then suspended in 100 mL dimethyl formamide (DMF) at 100°C for 30 min with constant agitation and then filtered on a glass filter. Approximately 25% of the total solids containing most of the colored material was solubilised. The DMF was then evaporated and the residue dissolved in 100 mL H_2O and refiltered before freeze drying. The remaining powder was dissolved in H_2O and centrifuged at 20,000 rpm for 10 min to discard any insoluble particles. The solution was then chromatographed on a Sephadex G25 (fine grade) column with water as eluent. Fractions of the first eluted peak were then pooled and freeze dried. A sample (100 mg) was dissolved in 10 mL of warm DMF and methylated overnight with $^{14}CH_3I$ (250 uCi) and K_2CO_3 (75 mg) with constant stirring. Excess

unlabelled CH_3I (50 µL) was then added and stirring continued for 4 hours to complete the methylation. The reaction mixture was evaporated, redissolved in 10 mL H_2O and centrifuged. The solution was then chromatographed on G-25 as described previously. The radioactive fractions of the first eluted peak were pooled and freeze dried to give 78 mg of radiolabelled, high molecular weight, chlorinated lignin (C14-BKE) with a specific activity of 212,000 DPM/mg.

Assay for C14-BKE Degradation to $^{14}CO_2$

Each reaction mixture contained 16 mg/mL of microorganism (based on dry weight determination), 0.5 mg/mL (106,000 DPM/mL) of radiolabelled BKE, 10 mM glucose in a medium that contained (L^{-1}): KH_2PO_4, 0.8 g; K_2HPO_4, 0.2 g; $MgSO_4.7H_2O$, 0.89 g; $NaNH_4HPO_4.4H_2O$, 0.2 g; urea 51 mg; $ZnSO_4.7H_2O$, 4.4 mg; $MnSO_4.H_2O$, 3.8 mg; $CuSO_4.5H_2O$, 1 mg; ferric citrate, 5 mg; $CaCl_2$, 20 mg. The medium was buffered at pH 5.0 with 2,2-dimethylsuccinate, 0.05 M. A blank without microorganisms was also included. Erlenmeyer flasks (50 mL), containing 5 mL of the reaction mixture, were closed with rubber stoppers equipped with tube ports to permit periodic oxygen flushing and collection of CO_2 essentially as described earlier[10]. Flasks were then incubated for 20 days at 30°C with constant shaking (150 RPM). At 3 to 4 day intervals the flasks were cooled down in an ice bath to minimize evaporation, and flushed with sterile O_2 (50 mL/min) for 10 min. Effluent gas was first passed through a weakly acidic solution, and CO_2 was then trapped in 10 mL of Carbon 14 Scintillation Cocktail (Cat. No. OX 161, R.J. Harvey Instrument Corp.). Radioactivity was measured with a Beckman LS 6800 Liquid Scintillation Counter. The efficiency of counting was determined by the sample counts ratio and calibrated with ^{14}C quenched external standards (New England Nuclear).

Distribution of ^{14}C and Colour

After the incubation period of 20 days, the contents of the

reaction vials were centrifuged to separate cells from supernatant. Cells were washed twice with 5 mL of the above medium and the supernatants pooled together. The cells were resuspended and centrifuged with (25 mL) 0.1 M NaOH, and finally with 5 mL of water. Alkaline and water supernatants were pooled and the remaining cells were combusted in a biological oxidizer (R.J. Harvey Model OY 400). The resulting $^{14}CO_2$ was collected and counted as described above. Radioactivity and colour were determined in each supernatant and compared with a blank without microorganisms. Radioactivity was determined in a HP/b scintillation cocktail (Beckman) and colour was measured as optical density at 465 nm after adjusting the pH to 7.6[2].

Molecular Weight Profile of C14-BKE Remaining in Solution

The supernatant from the original cultures (above) (100 µL) was submitted to gel permeation chromatography on a series of I-125 + I-60 columns (Waters Assoc.) using 0.05 M sodium phosphate buffer, pH 6.5, as mobile phase. Fractions of 1 mL were collected in scintillation vials and counted for radioactivity.

Time Course Reactions at Low Biomass Concentration

Reaction mixtures were essentially the same as described in the assay for C14-BKE degradation to $^{14}CO_2$, except that the biomass concentration was lowered to 2 mg/mL in a total reaction volume of 2.5 mL. Aliquots of 400 µL were taken at different times and centrifuged to separate cells from supernatant. Radioactivity and colour were determined as described above. Following the experiment, cultures were plated to determine viability.

RESULTS

Distribution of ^{14}C at High Biomass Loading

The rate of $^{14}CO_2$ production from ^{14}C-methylated, high molecular weight, chlorinated lignin (C14-BKE) in a nitrogen-

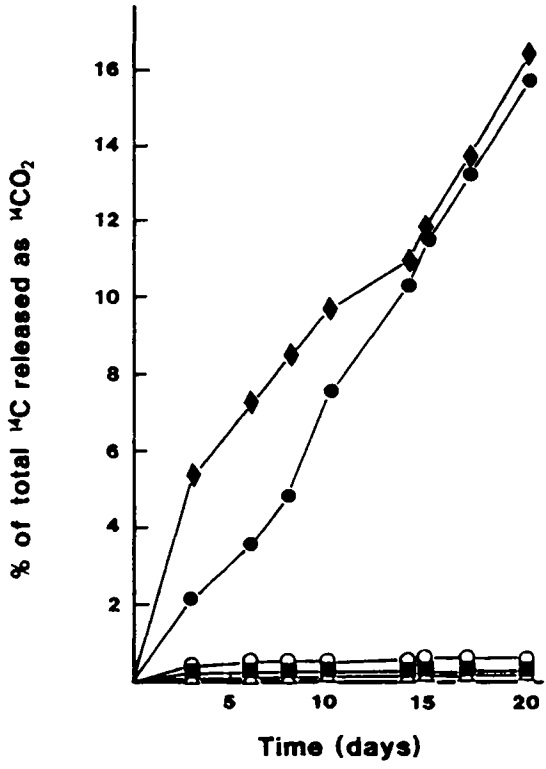

FIGURE 1: Release of $^{14}CO_2$ from C14-BKE in cultures of white-rot fungi and bacteria. The C14-BKE was supplemented with nutrients (see Materials and Methods), buffered at pH 5, and $^{14}CO_2$ was collected from shaken cultures by flushing with oxygen. Supplemental glucose (10 mM) was added to all cultures at day 14. Fungi: Coriolus versicolor (◆), Pleurotus sajor-caju (●). Bacteria: Pseudomonas aeruginosa ATCC 31482 (○), Bacillus cereus (△), and Pseudomonas aeruginosa SR13 (■).

limited medium at an initial biomass concentration of 16 mg/mL is shown in Figure 1. The two fungi Pleurotus sajor-caju and Coriolus versicolor were capable of mineralizing the substrate, producing about 16% conversion to $^{14}CO_2$ after 20 days incubation. Addition of glucose (10 mM) on day 14 increased the rate of $^{14}CO_2$ production with C. versicolor, but not signifi-

cantly with the other microorganisms. In fact, none of the bacteria tested produced significant amounts of $^{14}CO_2$ under our experimental conditions, which were established for efficient decolorization.

The distribution of ^{14}C between gas, liquid and solid components of the cultures after 20 days is summarized in Table 1. Gas production in a blank (no microorganism) was negligible. Cell-incorporated material was distinguished from cell-adsorbed by washing the cells with sodium hydroxide. The distribution of colour is also shown. For C. versicolor, most of the ^{14}C (62%) stayed in solution, even though 66% of the colour was removed. Very little of the ^{14}C or colour was associated with the cell, which indicates that the fungus modifies the chromophore in solution, without complete mineralization or incorporation into the cell. The ^{14}C distribution with P. sajor-caju was significantly different. While some ^{14}C and colour remained in solution, nearly half of the label was incorporated into or adsorbed onto the cells. For all three bacteria, the majority of both ^{14}C and colour was simply adsorbed on the surface, as shown by the high ^{14}C count and colour released by NaOH washing. However, a relatively high content of ^{14}C compared to colour in solution suggests some modification of coloured material.

Molecular Weight Changes in C14-BKE

Further evidence for metabolism was found when the molecular weight profiles of C14-BKE remaining after microbial treatments were compared with non-inoculated controls. For the fungal treatments (Figure 2a), lower molecular weight material was produced, although the distributions differ. All three bacteria produce a small amount of low molecular weight ^{14}C-labelled metabolite (Figure 2b) and adsorb on their cell surfaces practically all of the high molecular weight substrate. Most (92%) of the low molecular weight ^{14}C-labelled component could be removed by evaporation of samples to dryness.

TABLE 1

Distribution of ^{14}C and Colour Following 20-Day Incubation at pH 5.0 and 30°C of ^{14}C-BKE With Fungi and Bacteria.

Microorganism	% $^{14}CO_2$	Supernatant		Cell Adsorbed*		Cell Incorporated**	% Total
		% ^{14}C	% Colour	% ^{14}C	% Colour	% ^{14}C	^{14}C Recovery
FUNGI							
Coriolus versicolor	16.4	62.1	33.5	12.0	9.7	2.5	93.0
Pleurotus sajor-caju	15.7	29.9	20.6	27.6	18.6	20.8	94.0
BACTERIA							
Pseudomonas aeruginosa, ATCC 31482	0.7	16.0	8.7	62.5	78.4	13.5	92.7
Bacillus cereus	0.2	11.4	3.6	64.5	87.3	19.5	95.6
Pseudomonas aeruginosa, SR13	0.3	9.7	4.3	66.4	79.2	20.1	96.5

* Washed off with 0.1 M NaOH

** Determined by combustion of NaOH washed cells

Relationship Between ^{14}C and Colour Removing Capacities

To examine further the relationship between the rates of ^{14}C removal and colour decrease in solution, lower biomass concentrations (2 mg/mL) were used. This allowed the rates to be determined for fungal (Figure 3a) and bacterial (Figure 3b) cultures. The extent of colour removal by the two fungi was approximately equal, reaching over 70% after 210 hours. However, for C. versicolor, and to a lesser extent P. sajor-caju, much of the ^{14}C remained in solution, indicating, as in Table 1, that changes occur in the chromophore without mineralization. In the case of bacterial treatments (Figure 3b), SR-13 clearly showing the highest adsorption capability in terms of both colour and ^{14}C.

DISCUSSION

Radiolabelling of the high molecular weight compounds from bleached kraft effluent by direct methylation with ^{14}C-methyl iodide is relatively fast and inexpensive. The radiolabelled product was decolorized by the fungi and bacteria selected here, which can also decolorize unlabelled BKE. Other radiolabelled BKE substrates, prepared by kraft cooking and bleaching of ^{14}C-DHP[13] and [lignin-^{14}C] lignocellulose[14] have been successfully utilised but are much more difficult to prepare.

The utility of the C14-BKE for distinguishing between microorganisms capable of true metabolism and those simply adsorbing the substrate is demonstrated in the case of Pseudomonas aeruginosa (ATCC-31482). This microorganism, which is the subject of a patent claim for decolorizing pulp and paper mill effluent[4], removes colour at pH 5 mainly by adsorption without significant mineralization (Table 1). Thus, unless the bacteria are growing rapidly, the colour-removing ability will be limited to their adsorptive capacity. Since pulp and paper mill effluents are low in nitrogen, thus preventing rapid growth, one can predict

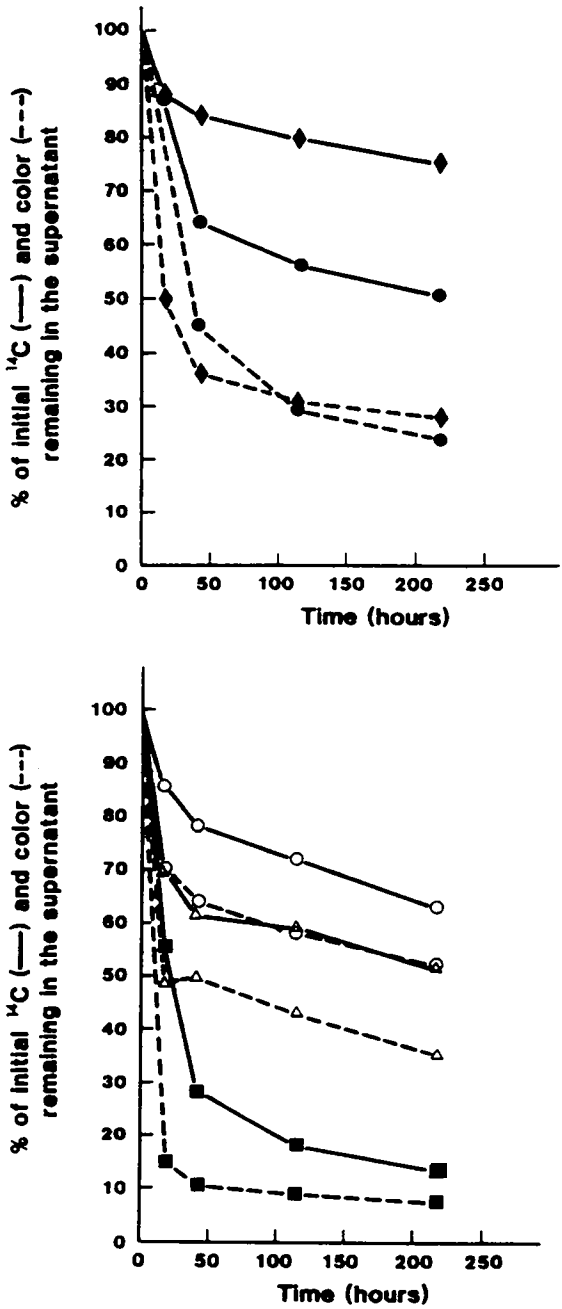

FIGURE 2: Gel permeation chromatograms of C14-BKE before (dashed line) and after (solid lines) treatment with (a) white-rot fungi and (b) bacteria. Symbols described in Figure 1.

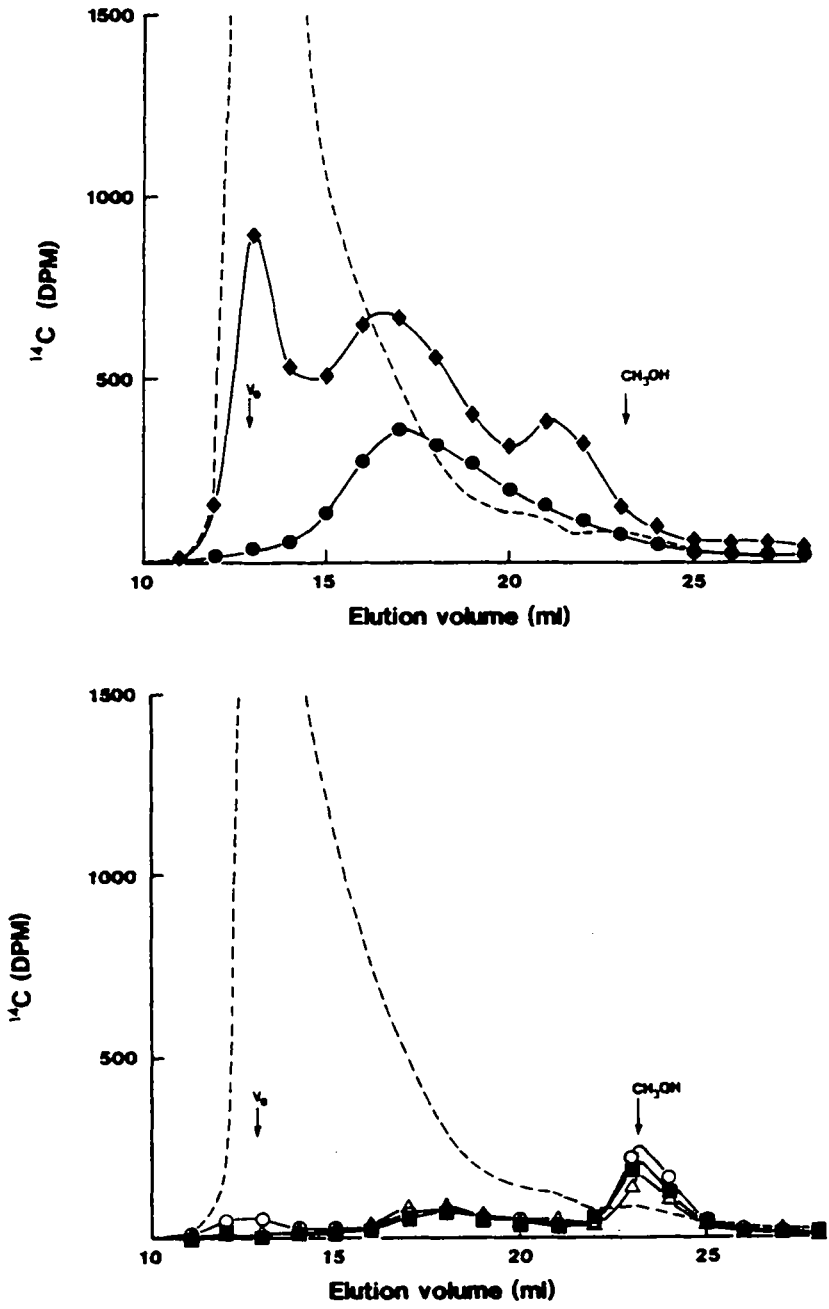

FIGURE 3: Removal of colour (dashed lines) and ^{14}C (solid lines) from solution by (a) white-rot fungi and (b) bacteria, at initial biomass loading of 2 mg/mL. Symbols described in Figure 1.

that sustained colour removal will not be possible without high nutrient addition, and this was found to be the case in industrial trials[16].

All three bacteria produced small quantities of a low molecular weight ^{14}C-labelled metabolite (Figure 2b) which, on the basis of volatility and retention time on gel permeation, appears to be methanol. Thus, the bacteria may be capable of limited demethylation or demethoxylation. The fungi produced both $^{14}CO_2$ and low molecular weight radiolabelled metabolites. Sundman et al.[17] have already shown that decolorization by P. chrysosporium is due to a combination of destruction of chromophores in the polymer and decomposition of the polymer to low-molecular-weight products. Our observation of $^{14}CO_2$ production by fungi, and lack of production by bacteria, is in agreement with that of Eriksson[14]. However, the major pathway to colour removal by C. versicolor and P. sajor-caju does not involve mineralization (Table 1 and Figure 3a). For C. versicolor, the majority of label remains in solution even when 75% of the colour is removed (Figure 3a). The ^{14}C distribution for P. sajor-caju is different. More of the label is found to be cell bound than in C. versicolor. However, relative to the bacteria, much less of the cell-bound material can be washed off with NaOH.

Mineralization of high molecular weight chlorinated lignin by C. versicolor is stimulated by glucose (Figure 1). This is in agreement with the proposal that this fungus removes colour, at least in part, through hydrogen peroxide production from glucose oxidase[8]. P. chrysosporium also requires a readily metabolizable carbon source for decolorization of BKE[3], and glucose addition also stimulates ligninolytic activity ($^{14}CO_2$ production from ^{14}CDHP) in nitrogen-limited cultures[18], possibly through the mediation of glucose oxidase[19].

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