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Enhanced removal of CCA from treated wood by *Bacillus licheniformis* in continuous culture

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ENHANCED REMOVAL OF CCA FROM TREATED WOOD BY *BACILLUS LICHENIFORMIS* IN CONTINUOUS CULTURE

by

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SUMMARY

A gram positive, spore-forming bacterium, which was isolated from a 20-year-old Forest Service test plot of CCA-treated 2 x 4's in Madison, WI., demonstrated the ability to release copper, chromium, and arsenic from CCA-treated wood in liquid culture. CCA-treated sawdust was exposed to this organism, which has been presumptively identified as *Bacillus* *licheniformis.* Analysis of the sawdust by atomic absorption spectroscopy revealed 80% reduction in copper and 43% reduction in arsenic when the sawdust was exposed to *B.* *licheniformis* in a standard mixed culture for 10 d compared to an untreated control. Enhanced release of metals was demonstrated when CCA-treated sawdust was exposed to the bacterium under continuous culture conditions in a chemostat. Steady-state growth of the bacterium under continuous culture conditions released 93% copper, 45% arsenic, and 6% chromium by weight from the sawdust. Exposure of CCA-treated wood to this isolate of *B.* *licheniformis* removed significant amounts of copper and arsenic from the wood.

Key Words: *Bacillus licheniformis,* **bacteria, CCA, preservative**

INTRODUCTION

Chromated-copper arsenate (CCA) has become the most popular water-born wood preservative in the U. S.; currently 600 million cubic feet of wood are treated with CCA in the U.S. annually (Micklewright, 1994). There has been a 300% increase in demand for treated lumber between 1980 and 1993 (Smith and Shiau, 1996). CCA-treated wood has an expected service life of 20-50 years depending on the conditions of service and the method of treatment (Copper, 1993; Smith and Shiau, 1996). Based on current levels of production and expected service-life, Copper (1993) predicts that by the year 2020, 16 million cubic meters of CCA-treated wood will be removed from service annually

in the U.S. Environmentally benign methods of disposal or recycling of the fiber source will need to be developed before the turn of the century. One possible method for recycling CCA-treated wood fiber would be to microbiologically remove chemical components and recycle both the wood fiber and the metals separately.

Gram-positive spore-forming bacteria from the genus *Bacillus* **are commonly isolated from preserved wood, possibly due to the resistance of bacterial spores to harsh conditions. They are tolerant of copper levels normally utilized to inhibit Basidiomycetous fungi, the group of fungi targeted by wood preservatives (Shields, 1969; Cole and Clausen, 1997). Fungal and bacterial tolerance of CCA varies with the amount of preservative present and the service condition (Singh *et al.*, 1994). Greaves (1973) looked at the intracellular uptake of CCA components by species of** *Bacillus, Pseudomonas,* **and** *Streptococcus* **isolated from preservative-treated wood. He found that CCA was bacteriostatic rather than bactericidal at concentrations as high as 0.675%. Singh *et al.*, (1994) presented microscopic evidence of erosion bacteria causing degradation in failed CCA-treated timbers. Attack by erosion bacteria was throughout the diameter of the pole, while attack by soft-rot fungi was confined to the outer areas of the pole.**

Cole and Clausen (1997) surveyed a Valley View test plot containing CCA-treated 2 x 4s in Madison, WI for bacteria that could tolerate CCA. It was determined that an isolate, presumptively identified as *Bacillus licheniformis,* **could release copper, chromium and arsenic from CCA-treated sawdust when the sawdust was exposed to the bacterium in a mixed culture for 3 wk. Intracellular accumulations of copper and chromium were also detected by energy dispersive x-ray analysis (EDAX). The objective of this study was to assess whether continuous culture with the** *B. licheniformis* **isolate would enhance removal of the metal components of CCA from treated wood in a shorter incubation time than the nutrient limiting conditions of a standard mixed culture.**

MATERIALS AND METHODS

Culture conditions and organism characteristics

Bacillus licheniformis **is a spore-forming gram positive rod. It is a facultative anaerobe that forms smooth, opaque colonies at 25°C. Cultures were maintained on nutrient agar (Difco) at room temperature.**

Mixed culture

Flasks (300 ml) containing 50 ml nutrient broth and 0.5 g CCA-treated sawdust (0.4 psf retention; ground to 20 mesh) were inoculated with 1 ml of an 18 h broth culture of *B. licheniformis.* Cultures were incubated at 25°C on a rotating table for 10 d. Controls consisted of uninoculated flasks of nutrient broth with sawdust that was either added before autoclaving or autoclave separately from the culture medium and added aseptically.

Continuous culture

The chemostat consisted of a series of flasks (Figure 1) which delivered fresh nutrient broth (A) drop by drop to a 2 L culture of *B. licheniformis* **containing 30g CCA-treated sawdust. The culture flask (C) was inoculated with 10 ml of an 18h broth culture of *B. licheniformis* and mixed at 200 rpm. Spent culture medium was collected in a third flask (D) for analysis. A total of 6.5 L of nutrient broth was added to the actively growing culture of** *B. licheniformis* **at 250°C over a 7 d incubation period.**

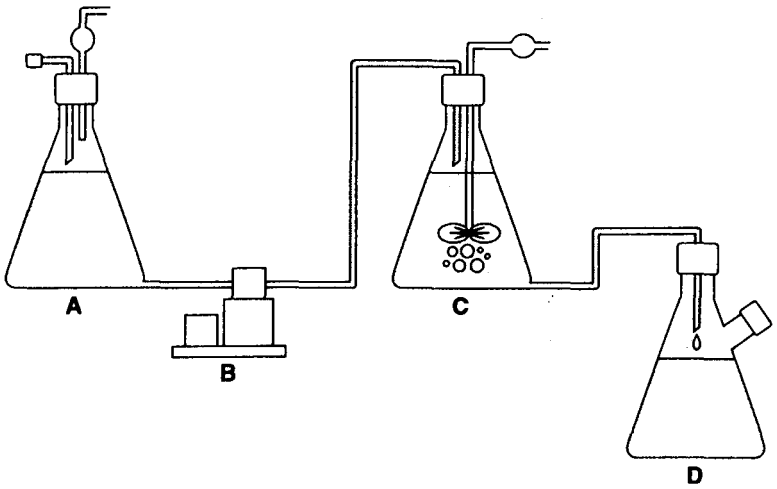

Figure 1. Chemostat design (A) sterile medium reservoir, (B) pump, (C) culture flask, (D) spent medium reservoir.

Sample collection

Bacterial cells were collected from the continuous culture spent medium reservoir by centrifugation at 16,000 x g for 30 min. Pelleted cells were resuspended in 40 ml water and autoclave to kill remaining viable cells. A 40 ml sample of pooled culture filtrate was removed for analysis and autoclave. Any remaining culture medium was removed from the sawdust by aspiration

through Whatman filter paper. The sawdust was rinsed briefly with deionized water and dried at 60°C for 24 h.

Controls from the mixed culture experiment showed that neither autoclaving nor exposure to mixing in sterile culture medium for 10 d reduced the amounts of CCA in treated sawdust. Thus, the continuous culture control consisted of CCA sawdust that was not exposed to culture medium or the bacterium.

Sample analysis

For the mixed culture experiment, 0.5 g samples of sawdust exposed to the bacterium were analyzed by atomic absorption (AA) according to AWPA (1993) to determine copper, chromium and arsenic levels. For the continuous culture experiment, 20 ml samples of ceils and culture filtrate and 0.5 g samples of treated and untreated CCA sawdust were analysed by AA.

RESULTS

The results of bacterial release of CCA from treated wood are shown in Table 1. Untreated CCA-sawdust at 0.4 psf retention analysed by AA showed control values of 2.28 mg/ g copper, 4.21 mg/ g arsenic and 3.80 mg/ g chromium. Standard mixed cultures exposed to *Bacillus licheniformis* **for 10 d showed a reduction of 79.8% copper and 42.8% arsenic compared to the control values. Negligible amounts of chromium were removed under standard mixed culture conditions. CCA-treated sawdust exposed to the bacterium in continuous culture using a chemostat demonstrated increased metal removal of 92.5% copper, 42.3% arsenic, and 6% chromium (by weight) compared to the mixed culture.**

Table 2 shows the results of analysis for all components of the continuous culture experiment (sawdust, cells and culture filtrate). Culture filtrate (spent medium) contained 28.80 mg copper, 32.40 mg arsenic and 4.80 mg chromium. Bacterial cells collected from spent culture medium accounted for 0.52 mg copper, 0.30 mg arsenic and 0.30 mg chromium of the total weight of CCA in the sawdust before bacterial exposure. Total recoveries of 50%, 99%, and 81% of total initial weight of copper, chromium, and arsenic, respectively were accounted for after continuous culture.

DISCUSSION

Removal of copper and arsenic from CCA-treated sawdust was enhanced when the sawdust was exposed to an isolate of *Bacillus licheniformis* **under steady-state continuous culture conditions using a chemostat. Continuous culture enables prolonged exposure to an organism with a rapid generation time by continually adding fresh medium to the culture while removing spent culture medium, thereby eliminating nutrient depletion. Compared to a standard mixed culture, an increase from 80% to 93% of the copper was removed, and an increase from 43% to 45% of the arsenic was removed (by weight) when using a chemostat to provide steady-state continuous culture conditions.**

Small amounts of chromium (6%) were removed under continuous culture conditions, while no chromium removal was demonstrated by bacterial exposure under mixed culture conditions. CCA is fixed to the lignin component of wood by the reduction of Cr^{+6} to Cr^{+3} (Hartford, 1986). The strong affinity chromium has for lignin (Zabel, 1992) aids in the retention of arsenic and copper, but the actual completing of chromium appears to inhibit it's removal in appreciable quantities by this isolate of *Bacillus.*

Members of both *Bacillus* **and** *Pseudomonas* **genera are ubiquitous in soils and produce pectinolytic and cellulolytic enzyme systems which may assist in releasing copper and arsenic from wood. Members of both genera are more resistant to copper than Basidiomycetes and have been isolated from preservative-treated wood (Greaves, 1973, Clausen, 1996). Tolerance levels of CCA in artificial medium showed that concentrations as high as 0.675% cause growth of these bacteria to cease, but the organisms remained viable (Greaves, 1973).**

Microscopic studies by Daniel and Nilsson (1985) and Daniel *et al.* (1987) indicated that copper was accumulated as dense particles in the nuclear region of unidentified tunnelling bacteria, while Cr and As were found in extracellular secretions. Energy dispersive x-ray analysis suggested that this isolate of *Bacillus licheniformis* **accumulated copper and chromium intracellularly (Cole and Clausen, 1996). However, bacterial cells collected from spent medium accounted for a small fraction of the total CCA by weight of the initial test material. Since most of the released metals were found in the spent culture medium, it is possible that this isolate of** *Bacillus* **enzymatically removed the metal components of CCA-treated wood and released them into the culture medium. The metals may be accumulated intracellularly long enough to be packaged in vacuoles to prevent toxicity to the organism prior to their release into the culture filtrate. Many questions on the mechanism of release of CCA from the wood remain. Extracellular enzymatic action as a mechanism of metal release will be explored**

in future studies. Preliminary studies indicate that this bacterium or a consortium of microorganisms may provide a microbiological method for removing the metal components from CCA-treated wood.

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Table 1. Atomic absorption analysis for copper, chromium, and arsenic from CCA sawdust following exposure to *Bacillus* *licheniformis* in standard mixed culture and steady-state continuous culture.

Treatment	mg/g CuO	percent reduction	mg/g CrO_3	percent reduction	mg/g As_2O_5	percent reduction
Untreated CCA sawdust	2.28		3.80		4.21	
Treated sawdust						
mixed culture	0.46	79.82	3.85	0	2.41	42.76
continuous culture	0.17	92.54	3.58	5.79	2.32	44.89

Table 2. Recovery of copper, chromium, and arsenic from sawdust, spent culture filtrate and cells after exposure of CCA sawdust to *B.* *licheniformis* in a chemostat.

Sample	Total mg Copper	% initial weight	Total mg Chromium	% initial weight	Total mg Arsenic	% initial weight
Original sawdust	68.40	100.00	114.00	100.00	126.30	100.00
Treated sawdust	5.10	7.46	107.40	94 21	69.60	55.11
Culture filtrate	28.80	42.11	4.80	4.21	32.40	25.65
Cells	0.52	0.76	0.30	0.26	0.30	0.24
Total recovery	34.42	50.33	112.50	98.68	102.30	81.00