
23 Bioremediation of Treated Wood with Fungi

Barbara L. Illman and Vina W Yang

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23.1 INTRODUCTION

Fungi have been investigated for the bioremediation of toxic chemicals since the 1980s, especially for the cleanup of industrial sites. Rare strains of *Basidiomycota* and *Ascomycota* have been shown to be tolerant to organopollutants and toxic metals. Early work on fungal remediation of wood preservatives focused on cleanup of wood treatment sites, with special emphasis on soil remediation as outlined by Lamar et al.¹ More recent studies have examined bacterial and fungal bioremediation of wood treated with metal-based preservatives. This chapter will introduce the remediation of treated wood with fungi and provide the results of work from the authors' laboratory to develop the technology.

DeGroot and Woodward conducted several extensive studies on the use of fungi for bioprocessing wood treated with copper-based preservatives.²⁻⁴ Using wood-decayfungi known to have copper tolerance, laboratory tests were conducted to determine degradation of sapwood treated with ammoniacal copper citrate (CC), ammoniacal copper quaternary (ACQ-B, ACQ-D), chromated copper arsenate (CCA), ammoniacal copper zinc arsenate (ACZA), or oilborne copper-8-quinolinolate or copper naphthenate (CuN). Low copper retention levels stimulated decay by *Wolfiporia cocos* but not *Postia placenta*. Copper-tolerant isolates of *W. cocos* were identified as potential agents for degrading wood treated with several copper-based preservatives.

The copper tolerant fungus *Antrodia vaillantii* shows promise as an agent for bioremediation of copper- and chromium- (CCA or copper chromium boron, i.e., CCB) treated wood⁵ and CCA-treated wood.^{6,7} The fungus produces high concentrations of oxalic acid in the decaying wood, causing an increased acidity that increases the solubility and subsequent removal of chromium and arsenic.^{7,8} The fungus significantly increased ammonia leaching of copper and chromium from CCA- and CCB-treated Norway spruce wood. Copper oxalate was identified in the leachate by electron paramagnetic resonance (EPR), supporting an important role for oxalic acid in copper tolerance.^{9,10} The EPR signal for Cu(II), Cr(V) and Cr(III) decreased in CCA- and CCB-treated wood that was colonized by copper-tolerant *A. vaillantii* or copper-sensitive *Poria monticola*.

The brown-rot fungi *Fomitopsis palustris*, *Coniophora puteana* and *Laetiporus sulphureus* have been evaluated for bioremediation of CCA-treated Scots pine wood chips.¹¹ Copper, chromium and arsenic were removed from the chips during submersion in 10-d-old liquid cultures of the fungi. The largest amount was removed by *F. palustris*: 100% of arsenic, 87% of chromium and 77% of copper. Decreasing concentrations of the metals correlated with the accumulation of high concentrations of oxalic in the culture medium, supporting an oxalic acid chelation mechanism. Another fungus known to secrete large quantities of oxalic acid, *Aspergillus niger*, was used in a two-step process to remove 97% of arsenic, 55% of chromium and 47% of copper from CCA-treated wood chips.¹²

The authors have developed technologies for fungal bioremediation of waste wood treated with oilborne or metal-based preservatives.¹³⁻¹⁸ The technologies are based on specially formulated inoculum of wood-decayfungi, obtained through strain selection to obtain preservative-tolerant fungi. This waste management approach provides a product with reduced wood volume and the capacity to collect metals for reuse, thereby preventing environmental contamination. In this chapter we describe our research on the bioremediation of CCA-treated wood waste with brown-rot wood-decayfungi. The paper contains new data and summarizes past research.^{13,19,20} The research includes (1) isolating and characterizing CCA-tolerant fungi, (2) defining economical materials and methods to prepare and package viable inoculum of metal-tolerant decay fungi, (3) establishing treatment procedures for the remediation and degradation process, and (4) conducting a laboratory scale-up to evaluate the method on solid lumber treated with CCA.

23.2 MATERIALS AND METHODS

23.2.1 WOOD DECAY FUNGI

Fungi were collected from field test sites or selected from the extensive fungal library at the Center for Forest Mycology Research, Forest Products Laboratory (FPL), Madison, WI (Figure 23.1). The FPL fungal library was screened for isolates (identified to species but not to strain) that were collected from preservative-treated wood products, such as decks, poles and test stakes that had visible signs of decay. Additional isolates were taken from 20- to 30-year-old preservative-treated wood stakes taken from the FPL field test plots in Gulfport, MS and Picnic Point, Madison, WI. Fungi were cultured on 2% malt extract agar (MEA, DifcoBacto™) or a modified Taylor's medium at 27°C and 70% relative humidity. Mycelium was stored on 2% malt extract agar test tube slants or in petri dishes at 4°C.

23.2.2 METAL TOLERANCE ASSAY

Fungal tolerance to CCA was determined by two methods, an *in vitro* bioassay described as a “choice test” by Leithoff et al.²¹ and a growth response bioassay of *in vitro* exposure to metals. Briefly, for the choice test assay a 9-mm fungal disk from a freshly grown malt agar culture was placed in the center of a petri dish (14 cm in diameter) containing 12 ml of a 2% agar medium with a CCA-treated and a untreated Southern yellow pine wood sample (1.5 × 0.3 cm) placed at opposite edges of the plate. Fungi were kept in an incubator at 27°C and 70% relative humidity for 14 d and were observed for growth response to the treated wood. Metal tolerance was rated as fungal growth toward or on the treated wood. For the metal exposure assay, copper,



FIGURE 23.1 Collecting field stakes to isolate fungi.

chromium or arsenic were incorporated into 2% MEA petri plates and inoculated in the center with *Meruliporia incrassata* (TFFH-294) and *Meruliporia incrassata* (TFFH-295). Cupric sulfate, potassium dichromate or sodium arsenate (Aldrich) were added to MEA at levels of 0, 0.1, 1, 10 and 100 mM of Cu, Cr or As. Mycelium was measured from the center of the plate on Day 14 to determine metal effect. Response was expressed as percentage of growth without metals in the medium.

23.2.3 OPTIMUM GROWTH CONDITIONS

Temperature for optimum growth was determined by an *in vitro* assay. Four disks (9 mm) of freshly grown fungal cultures were removed from 2% MEA plates and inoculated into 125-ml Erlenmeyer flasks containing 25 ml of 2% malt extract liquid medium (DifcoBacto). Flasks were placed in an incubator at 20, 27, 32 or 37°C at 70% relative humidity in the dark for 12 d. Mycelium was harvested by straining the liquid culture through previously weighed Whatman No. 1 filter paper, allowing the mycelia to air dry on the paper, reweighing and calculating biomass dry weight.

Light conditions for optimum growth were determined for liquid cultures as described above for temperature. Flasks were kept stationary in an incubator at 27°C and 70% relative humidity for 12 d under one of the following light regimens: 24 h of light, 12 h of light with 12 h of darkness, or 24 h of darkness.

The effect of oxygen and chemically defined media on fungal growth was determined for liquid cultures. Four disks (9 mm) of freshly grown fungal cultures were removed from 2% MEA plates and inoculated into 125-ml Erlenmeyer flasks containing 25 ml of Bailey's medium²² or BIII medium²³. Flasks were kept stationary in an incubator at 27°C and 70% relative humidity in the dark for 21 d, with or without an exposure to a 20-sec oxygen flush on alternate days. Mycelia were harvested and biomass dry weight determined as above with temperature and light. There were three replicate flasks per medium per oxygen treatment.

23.2.4 FUNGAL INOCULUM

Nutrient supplements were tested for effects on fungal growth. Disks (9 mm) of freshly grown fungal cultures were removed from 2% MEA culture plates and inoculated into 125-ml Erlenmeyer flasks containing 25 ml of 2% malt extract liquid medium supplemented with or without 1% sterile corn steep liquor (CSL) from corn processing (ADM, Cedar Rapids, IA). Flasks were kept stationary in an incubator at 27°C and 70% relative humidity for 3 weeks. Mycelium was separated from liquid culture by filtration through previously weighed Whatman No. 1 filter paper, allowed to air dry on the paper and reweighed. Dry weight of CSL-fed mycelium was expressed as percentage dry weight of mycelium grown without CSL.

Lignocellulose substrates (sawdust, wood chips, rice straw, corn stalks and wheat straw) were evaluated for effectiveness as long-term food sources in the fungal inoculum and as a matrix for inoculum storage and handling. Similar methods apply to all. Sawdust and wood chips were steam-sterilized and cooled at room temperature prior to mixing with the fungal nutrient supplement mixture.

Steam sterilization of the lignocellulose substrate is preferred as the steam provides both sterility and moisture content. Moisture from steam-sterilization enhances fungal growth.

23.2.5 WOOD DEGRADATION

Blocks of Southern yellow pine ($1 \times 1 \times 0.3$ in.) were treated with CCA to 6.4 kg/m^3 according to American Wood Preserver's Association standards.²⁴ Treated blocks were inoculated with *Meruliporia incrassata* (TFFH-294), *Antrodia radiculosa* (MJL-630), *Meruliporia incrassata* (Mad-563) or *Antrodia radiculosa* (FP-90848-T) according to the ASTM standard soil bottle decay test.²⁵ Briefly, a CCA-treated block ($2.5 \times 2.5 \times 0.9$ cm) was inoculated with fungi in soil bottles, incubated for 12 weeks at 27°C and 70% relative humidity, and weight loss was determined. Treated and untreated control blocks were incubated without exposure to fungi. The test was replicated five times. Decay was expressed as percentage weight loss.

A fungal inoculum amended with nutrients and lignocellulose supplements was prepared with minor modification of the method described in the section above. Fungal mycelium was transferred from stock culture to 10 ml of 2% MEA in a glass bottle ($2 \times 2 \times 5$ in.) and incubated in the dark at 27°C and 70% relative humidity for 2 weeks. The resulting mycelium was mixed in the bottle with 10 g of sterile sawdust, 20 ml of sterile water, and 20 ml of sterile 1% CSL or 0.25 g of a 50:50 mixture of sterile wheat bran and cornmeal, incubated in the dark at 27°C and 70% relative humidity for 6 weeks.

23.2.6 LABORATORY SCALE-UP

The inoculum preparation described in the sections above was modified to evaluate the effectiveness of the method on larger volumes of solid lumber and particulate, flaked or chipped CCA-treated Southern yellow pine. Sawdust (350 g) was sterilized in an aluminum tray ($22.9 \times 33.1 \times 6.4$ cm) cooled to room temperature and mixed with 700 ml of 1% CSL. *Meruliporia incrassata* (TFFH-294) was cultured in seven petri dishes (14 cm in diameter) containing 2% MEA, incubated at 27°C and 70% relative humidity for 2 weeks. The resultant culture was cut into pieces (approximately 3.8-cm square), mixed with the solid substrate in the tray, incubated at 27°C , 70% in the dark for 8 weeks. If not used for processing right away, the inoculum was stored at 4°C .

The bioprocessing method was evaluated in a laboratory scale-up with CCA-treated and untreated Southern yellow pine lumber. Several large metal chambers ($83.8 \times 15.3 \times 20.3$ cm) with sliding covers were custom-made for the decay test on lumber. A 5.1-cm layer of moistened soil with a water content of 35% lined the bottom of the chamber. Test samples of CCA-treated and untreated wood ($5.1 \times 10.2 \times 30.5$ cm) were placed on top of the soil and steam sterilized. After the chambers had cooled to room temperature, the wood was completely covered with the *Meruliporia incrassata* TFFH-294 inoculum. The closed chamber was placed in the dark in an incubator at 27°C and 70% relative humidity for 12 weeks. Weight loss of wood was calculated as described for the decay test above (Figure 23.2).

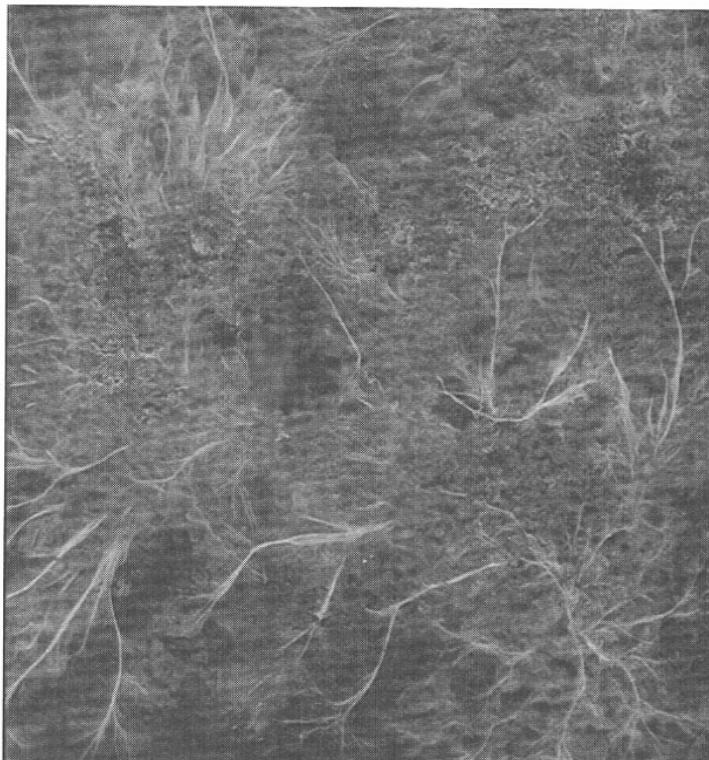


FIGURE 23.2 *Meruliporia incrassata*.

23.3 RESULTS

23.3.1 WOOD DECAY FUNGI

A total of 150 brown- and white-rot wood-decayfungi were obtained from metal-treated wood. The isolates that exhibited CCA tolerance in the choice test are listed in Table 23.1. Most fungi grew toward untreated wood with no growth toward CCA-treated wood. The 18 fungal isolates in Table 13.1 grew toward and/or on CCA-treated wood. The brown-rot fungus, *M. incrassata* (TFFH-294) exhibited the most tolerance and was selected for the growth-response studies. Two isolates of *M. incrassata* were tolerant to 1 mm copper, chromium and arsenic (Table 23.2).

The response of *M. incrassata* (TFFH-294) to temperature and light was the same as reported for most wood-decay fungi known to be metal sensitive.²³ The optimum temperature range for *M. incrassata* (TFFH-294) biomass production was between 27 and 32°C with declining production at the higher and lower temperatures of 35 and 20°C (Table 23.3). Light inhibited fungal growth (Table 23.3). The fungus produced 33% more biomass when incubated in the dark for 24 h/d than for 24 h/d in light. *M. incrassata* (TFFH-294) exhibited a similar growth response on chemically defined medium and exposure to oxygen as that reported for many brown-rot fungi and for the white-rot fungus *Phanerochaete chrysosporium* (FPL-GTR-113).^{23,26} Biomass production was slightly higher on Bailey's than on BIII medium, and production was enhanced on both media when exposed to oxygen (Table 23.4).

TABLE 23.1
Wood Decay Fungi Tolerant to CCA^a

Species	Isolate	Collection
<i>Antrodia radiculosa</i>	L-11659-sp	FPL-MC ^b
<i>Gloeophyllum</i> <i>subferrugineum</i>	FRI 417/R	FPL-MC
<i>Polyporus</i> sp.	FP134933	FPL-MC
<i>Trichaptum</i> <i>byssogenum</i>	FP105308-R	FPL-MC
<i>Gloeophyllum trabeum</i>	Boat 228	FPL-MC
	TLH-1	FPL ^c
<i>Antrodia radiculosa</i>	MJL-630	FPL-MC
<i>Neolentinus lepideus</i>	HHB 1 3625	FPL-MC
<i>Antrodia xantha</i>	MB268	FPL-MC
	CAC-1	FPL
	P6G	FPL-pp ^d
	F71H	FPL-pp
	UpK	FPL
	UpL	FPL
<i>Meruliporia incrassata</i>	FFH-294	FPL
<i>Meruliporia incrassata</i>	Mad-563	FPL
<i>Antrodia radiculosa</i>	FP-103272-sp	FPL-MC
<i>Antrodia radiculosa</i>	FP-90848-T	FPL-MC

^aCCA tolerance determined by choice test.

^bFPL-MC Forest Products Laboratory Center for Forest Mycology Research.

^cFPL-RW4502 Forest Products Laboratory Biodeterioration Unit.

^dFPL-PP Forest Products Laboratory Picnic Point.

TABLE 23.2
**Growth Response of *Meruliporia incrassata* TFFH-294 and
MAD-563 on Malt Extract Agar Amended with Copper,
Chromium or Arsenic**

	Inhibition of growth (%)									
	<i>M. incrassata</i> (TFFH-294)					<i>M. incrassata</i> (MAD-563)				
Metal (mM)	0	0.1	1.0	10	100	0	0.1	1.0	10	100
Copper	0	0	0	100	100	0	0	0	100	100
Chromium	0	0	0	100	100	0	0	0	100	100
Arsenic	0	0	13.3	80.3	100	0	0	0	100	100

TABLE 23.3**Meruliporia incrassata (TFFH 294) Response to Light**

Light Condition	Weight (mg) ^a
24 h of light	66 ± 8
12 h of light	70 ± 6
24 h of dark	88 ± 13

^a ± Standard error

23.3.2 FUNGAL INOCULUM

Fungal growth and biomass production were enhanced by the addition of 1% CSL to the growth medium, but were adversely affected by higher concentrations (Table 23.5). Lignocellulose provided a solid, organic matrix for the fungal inoculum.

23.3.3 WOOD DECAY

Four isolates, *Meruliporia incrassata* (TFFH-294) *Antrodia radiculosa* (MJL-630), *Meruliporia incrassata* (mad-563) and *Androtia radiculosa* (FP-90848-T), degraded the CCA-treated wood more than 20% of the original dry weight of the wood (Table 23.6). Medium supplements enhanced degradation of CCA-treated wood (Table 23.7).

23.3.4 LABORATORY SCALE-UP

M. incrassata (TFFH-294) degraded CCA-treated lumber by 28%. Fungal growth was slower on treated as compared to untreated wood, but mycelia were clearly visible on the outer surface and interior of the wood (Figure 23.3).

23.4 DISCUSSION

A method for fungal degradation and/or bioremediation of CCA-treated wood waste has been developed, providing a product with reduced waste volume and the capacity for reuse of waste metals. An inductively coupled plasma mass spectrometry analysis

TABLE 23.4**Meruliporia incrassata (TFFH-294) Growth Response to Oxygen and Culture Medium**

Medium	O ₂	Weight (mg)	pH
Bailey	+	23 ± 1	2.73
	-	20 ± 1	3.04
BII	+	21 ± 2	2.96
	-	18 ± 2	3.61

TABLE 23.5
Effect of Corn Steep Liquor on Biomass Production of Metal-Tolerant
***Meruliporia incrassata* (TFFH-294)**

CSL Concentration (%)	% Dry Weight ^a
0	100
1.0	321
2.5	256
5.0	196

^aPercentage dry weight of mycelium grown without corn steep liquor.

of residual metals in the wood waste will be reported elsewhere. Early results indicate that residual metal concentrations decrease in the wood during bioprocessing, making it possible to collect the metals for reuse or remove the metals to prevent environmental contamination.

TABLE 23.6
Fungal Degradation of Preservative-Treated Wood

Fungal Species	Untreated	CCA
	Weight (mg)	
	Average (Standard Deviation)	Average (Standard Deviation)
<i>Meruliporia incrassata</i> (TFFH-294)	62.2 (2.9)	36.8 (2.7)
<i>Antrodia radiculosa</i> (MJL -630)	32.6 (4.8)	26.6 (2.9)
<i>Meruliporia incrassata</i> (Mad-563)	62.5 (2.5)	23.7 (3.2)
<i>Antrodia radiculosa</i> (FP-90848-T)	39.5 (4.1)	20.1 (7.7)
<i>Antrodia radiculosa</i> (FP-103272-sp)	24.6 (6.0)	6.5 (4.7)
<i>Antrodia radiculosa</i> (FP-105309-R)	27.2 (3.0)	2.3 (0.8)
<i>Antrodia radiculosa</i> (L-11659-sp)	23.1 (2.7)	1.3 (1.3)
<i>Neolentinus lepideus</i> (Mad-534)	38.8 (5.3)	0.7 (0.4)

Note: Method ASTM D-1413-76 Standard Method of Testing Wood Preservatives by Laboratory.

Source: Soil-block cultures from Reference 25.

TABLE 23.7
Effect of Inoculum Supplements on *Meruliporia incrassata* TFFH 294
degradation of CCA-treated wood

Fungal Species	Isolate	Inoculum Supplements		
		No Supplement ^a Weight (mg)	CMWB ^b	CSL ^c
<i>Antrodia radiculosa</i>	L-11659	32.0	150%	446%
<i>Meruliporia incrassata</i>	TFFH 294	34.0	32%	208%

^aInoculum with no corn meal and wheat bran or corn steep liquor.

^bCorn meal and wheat bran amended inoculum.

^cCorn steep liquor amended inoculum.

Unique, natural mutants of wood-decay fungi were taken from FPL collections or isolated from field test samples, characterized and reintroduced into CCA-treated wood to determine if they degraded wood, testing Koch's postulates. Fungal isolates identified as CCA tolerant and as degraders of CCA-treated wood include *Meruliporia incrassata* (TFFH-294), *Antrodia radiculosa* (MJL-630), *Meruliporia incrassata* (Mad-563), *Antrodia radiculosa* (FP-90848-T), and *Antrodia radiculosa* (L-11659).

An advantage of the bioprocessing method is that the CCA-tolerant fungi do not require genetic alteration to grow in the presence of and degrade CCA-treated wood. Thus, the introduction of the fungi into the environment provides no new, nonnaturally occurring organisms. However, the method is designed for contained waste-management facilities where fungal viability and growth can be altered with temperature, water, oxygen, light, nutrients and other conditions. Metal-tolerant

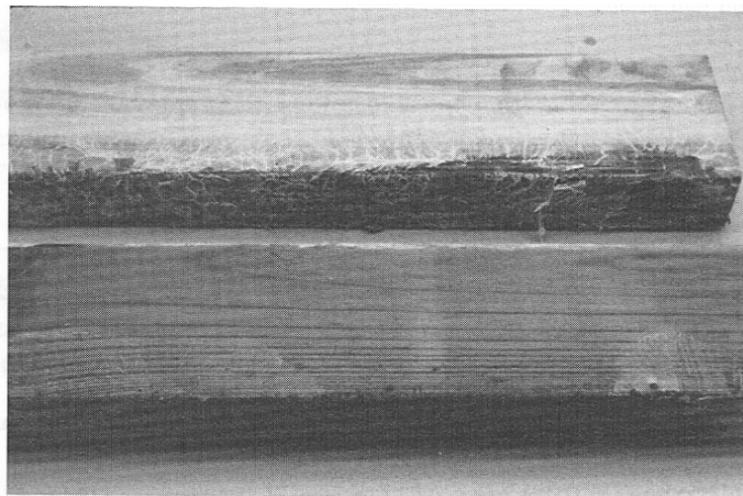


FIGURE 23.3 *Meruliporia incrassata* (TFFH 294) decay of CCA-treated wood. Top. Lumber is untreated Southern yellow pine. Bottom. Treated with CCA.

isolates such as *M. incrassata* (TFFH 294) thrive only in optimum conditions. It is a slow-growing isolate that would not be expected to compete in nature with faster growing microorganisms such as molds, bacteria or the decay fungi *Postia placenta* and *Gleophyllum trabeum*. Metal-tolerant *M. incrassata* (TFFH 294) has the advantage over other microorganisms during the bioprocessing because it has extensive mycelia growth in the inoculum and because competing microorganisms do not tolerate CCA on the lumber.

The inoculum in this method has many advantages. It is cost effective, utilizing agricultural waste products and waste products from sawmills and urban chipping. These products provide a quick and low-cost food source for the fungus, stimulate rapid and extensive fungal growth and provide a readily storable and transportable solid matrix. The inoculum should limit the disadvantage experienced by various fungal strains, such as *M. incrassata* (TFFH-294), that are typically disadvantaged by a slow growth that limits their competition with other dominant fungi in nature. The metal-tolerant strains can be easily grown as indicated by the optimum growth and nutrient conditions identified in this study. Fungal growth is substantially enhanced with aeration and nutrients added to the culture medium.

Another advantage is that the inoculum and its method of use are particularly well suited for wood waste such as pressure-treated lumber from buildings, decks, utility poles and railroad ties. The solid matrix of the fungal inoculum provides a wood environment for fungal growth that is similar to that of the wood waste. The fungal strain is, therefore, readily adapted to the waste wood on inoculation and does not require a period of adjustment before degradation and bioremediation begins.

The bioprocessing method is designed for remediating and degrading solid pieces of lumber, not costly chipping or flaking that may require environmental oversight in the future with increased concerns about worker exposure to airborne particles; it can be conducted on a small scale without costly transportation to larger, distant disposal sites; it is designed for a category of wood use that will not require extensive sorting, i.e., wood from docks, decks or landscaping can be kept separate more easily than mixed grades of wood from building demolition. The CCA-treated wood in this study was not wood waste; therefore, we expect higher yields with spent wood taken out of service.

A major component of the inoculum proved to be the lignocellulose substrate. Sawdust or wood chips are preferred for several reasons: (1) they provide a long-term food source for the fungus while providing fungal growth in a wood environment similar to the CCA-treated waste wood environment experienced during inoculation; (2) they permit the production of a large quantity of fungi in a single container; (3) they provide a substrate for easily storing and transporting the fungi; (4) they provide a matrix for convenient and even distribution of the fungus at the inoculation site; and (5) they provide a low-cost use of a waste product from sawmills.

Based on the success of the laboratory scale-up, the method promises to be successful in a pilot-level study in a larger waste facility. Several aspects of the procedure will need to be addressed for pilot-level scale-up. We predict that a higher percentage of wood degradation will be obtained with a longer incubation time. Potential contamination, especially mold contamination at later stages of the procedure,

warrants an evaluation of specific mildewcides to be added with the inoculum and/or applied during the incubation. Procedures for various sizes of lumber need to be identified and schedules developed for incubation on a pilot scale.

23.5 SUMMARY

An economical fungal bioprocessing method was developed as a waste-management system for CCA-treated wood taken out of service. The method reduces the volume of wood waste and provides the capacity for leaching and collecting the metals for reuse. A fungal inoculum is prepared by culturing a CCA-tolerant fungus on 2% MEA under aerobic conditions in the dark at a temperature range of 27 to 32°C and 70% relative humidity for 2 weeks; combining the fungal culture with a heat-sterilized mixture of 1% nutrient supplement (CSL), a lignocellulose substrate (sawdust) and water at 2 to 3 volumes per volume substrate; and incubating the mixture under aerobic conditions in the dark at a temperature range of 27 to 32°C and 70% relative humidity for 6 weeks. The inoculum can be transferred to bioprocessing containers or stored at 4°C. Large quantities of inoculum can be stored in trays or large durable plastic bags, loosely packed to allow aeration. Trays and bags can be transported easily to the field sites and applied to the wood waste. Inoculum can be prepared directly in a truck bed or in a truck-loaded container. A successful laboratory scale-up of the method provides support for further testing of the procedures in a pilot study at a larger waste facility.

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Edited by

Timothy G. Townsend
Helena Solo-Gabriele



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