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Oxalic acid production and metal removal during fungal degradation of CCA-treated wood in nutrient culture

Abstract: The objective of this study was to evaluate characteristics of oxalic acid (OA) production and metal removal during degradation of CCA-treated wood in nutrient culture by brown-rot fungi. Two brown-rot fungi, *Crustoderma* sp. and *Fomitopsis palustris* extensively degraded the CCA-treated wood, causing mass losses (MLs) up to 49.0% and 43.5%, respectively, while these fungi produced OA during degradation up to 21.3 mg g^{-1} and 43.8 mg g^{-1}, respectively. *Antrodia vaillantii* and *Polyporales* sp. produced OA up to 28.9 mg g^{-1} and 29.8 mg g^{-1}, respectively, with <3% ML. *Fomitopsis palustris* with the highest OA production removed effectively 87.5% As and 86.0% Cr during degradation of the treated wood. *Antrodia vaillantii* and an unknown *Polyporales* sp. showed notable As removal rates of 90.3% and 88.9%, respectively, and 81.0–83.9% Cr removal. However, only moderate amounts of Cu (40.8%) were extracted by the fungi investigated. The conclusion is that OA production by brown-rot fungi can be partially associated with removal of Cr and As during fungal degradation of CCA-treated wood.

Keywords: biodegradation, brown rot fungi, CCA-treated wood, metal removal, oxalic acid (OA)

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Introduction

The pretreatment of chromated copper arsenate (CCA)-treated wood wastes is a technical and economical challenge (Lin and Hse 2005; Hse et al. 2013). Fungal bioprocessing (biodegradation) is a cost-effective and environmentally sound alternative to alleviate problems caused by current disposal methods such as landfill and incineration. By means of this approach, volume/mass reduction and removal of metal components from the wastes can be achieved simultaneously (Illman et al. 2000; Clausen and Green III 2003; Choi et al. 2009, 2012a). Certain brown-rot fungi such as *Antrodia vaillanti* (DC.) Ryvarden, *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden, and *Meruliporia incrassata* (Berk. & M.A. Curtis) Murrill can break down polysaccharides in CCA-treated wood into monosaccharides in the course of their catabolism.

Subsequently, the strong chelating agent oxalic acid (OA) is synthesised via various metabolic pathways (e.g., via the tricarboxylic acid cycle), and Cr and As is chelated from CCA-treated wood (Munir et al. 2001; Kim et al. 2009). Brown rot fungi disposes of many chelating compounds, including catecholates and hydroxamates, and OA is only one of these compounds which involved metabolism and catabolism (Steenkjær Hastrup et al. 2006, 2013). OA secretion in this context was proposed as having a function in pH control and non-enzymatic biodegradation (Schilling and Jellison 2005).

Removal of metal components from CCA-treated wood wastes is necessary before disposal in landfills and incineration because of the adverse effects of Cr and As on human health and the soil and water environment; Cu components are especially detrimental in aquatic environments (Hamula et al. 2006; Barray et al. 2009). Contamination by metal leachate in this context is well documented (Saxe et al. 2007; Dubey et al. 2009). Before incineration, metal removal is mandatory to avoid fly ash contaminated with metals (Kakitani et al. 2004; Helsen and Van den Bulck 2005). On the other hand, volume/mass reduction of wood is disadvantageous for the energy balance when the waste wood is utilised by thermochemical conversion. Accordingly, an optimisation is needed concerning an effective metal removal and a gentle mass loss (ML) in the course of the fungal pretreatment (Kim et al. 2012). A number of studies describe brown-rot fungi leading to high ML and high metal removal rates during bio-treatment of CCA contaminated wastes. Recently, Choi et al. (2012a) demonstrated that five isolates of brown-rot

fungi, *A. vaillantii* SEL8501, *F. palustris* TYP0507 and TYP6137, and *Crustoderma* sp. KUC8065 and KUC8611, showed considerable degradability of CCA-treated wood with high removal rate of CCA components.

One of the essential questions of this topic is the interrelation between the OA production of the fungi and their ability of metal removal. However, this question could not be clarified in previous works because of the less suitable test methods applied (Curling et al. 2002). In particular, the presence of nutrients accelerates the production of OA during fungal degradation of wood (Micales 1994). The aim of the present study was therefore, to evaluate more reliably the characteristics of OA production and metal removal during fungal pre-treatment of CCA-treated wood in nutrient culture.

Materials and methods

Fungal isolates

Out of a total of seven brown-rot fungi, *A. vaillantii* SEL8501, *Crustoderma* sp. KUC8065 and KUC8611, *F. palustris* TYP0507 and TYP6137, *Gyrodontium sacchari* (Spreng.) Hjortstam GYV9595, and an unknown *Polyporales* sp. LAS6497, were the focus. All isolates, except for *Crustoderma* sp., were obtained from the Research Institute for Sustainable Humanosphere, Kyoto University, Japan, and the isolates notable tolerance to CCA was confirmed by the "choice test" described by Leithoff et al. (1995). Two isolates of *Crustoderma* sp., previously isolated from CCA-treated wood wastes (Choi et al. 2009), were obtained from the Korea University culture collection, Korea, and were also included in this study. The fungi were transferred from stock to 2% malt extract agar (MEA) media and maintained in the dark for 7–14 days at 27°C. The optimum growth temperature for decay testing was determined by comparing growth on 2% MEA media at 20°C–40°C (5°C intervals).

Preparation of wood samples

Logs of radiata pine (*Pinus radiata* D. Don), Western hemlock (*Tsuga heterophylla* Sarg.), and Japanese cedar (*Cryptomeria japonica* D. Don) were collected from the north of the South Island, New Zealand; Portland, Oregon, United States, and Jeju, Korea, respectively. The logs were sawn into 50 mm-thick boards and conditioned to approximately 15% MC. Sapwood blocks measuring 20 mm×20 mm in cross section by 10 mm in length were cut from air-dried board and stored at room temperature (r.t.) for more than 2 weeks.

The samples were vacuum-impregnated with CCA-C solution containing 18.5% CuO, 47.5% CrO_3, and 34.0% As_2O_5 to the target retention of 4.0 kg m^{-3}. Immediately after treatment, the samples were wrapped in plastic bags and stored for 2 days at 60°C to allow complete fixation of the CCA components. After fixation, the samples were air-dried at r.t. for more than 7 days. Untreated samples were also prepared from air-dried boards for comparison.

CCA retention was analysed after the samples reached constant weight. Samples were ground to fine sawdust in a Wiley mill and digested by the peroxide-nitric acid method according to American Wood Protection Association (AWPA) A07-04 (AWPA 2005a). CCA elements in digested solution were analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) according to AWPA 21-00 (AWPA 2005b). The initial CCA retention of the treated samples was then calculated based on their air dry density [*P. radiata* (509.3 kg m^{-3}), *T. heterophylla* (432.4 kg m^{-3}), and *C. japonica* (410.5 kg m^{-3})], as shown in Table 1.

Decay test

The degradability of wood samples was evaluated according to JIS standard method K 1571 (JSA 2004) with minor modification. Ten fungal disks (5 mm diameter) of each test isolate were removed from the pre-cultured 2% MEA media and inoculated into a flask containing 100 ml of nutrient solution (4% glucose, 1.5% malt extract, and 0.3% peptone). The flasks were agitated at 150 rpm on a rotary shaker for 7 days at 27°C. After incubation, the fungal mycelia were homogenised and 5 ml of fermentation solution was uniformly sprayed on sea sand in culture bottles. Prior to inoculation the culture bottle, which contained 250 g of sea sand and 71 ml of the nutrient solution (4% glucose, 1.5% malt extract, and 0.3% peptone), was steam-sterilised for 30 min at 121°C. To avoid direct contact between the wood samples and sea sand, plastic mesh was placed on the sand. The culture bottles were incubated for 2–3 weeks until the surface of the sand was completely covered by fungal mycelia. Then, three wood samples were placed on the plastic mesh. All bottles were incubated for 12 weeks at the optimum growth temperature for each fungal isolate.

Following incubation, the wood samples were removed from the culture bottles, brushed free of mycelia, dried at 60°C to constant weight, and weighed again to determine ML by comparing to the initial mass. All processes were performed under aseptic conditions; each isolate and sample was tested in triplicate.

Oxalic acid (OA) assay

After the 12-week decay test, the amount of OA produced by fungal isolates in the decayed blocks was quantified. To extract OA from the decayed blocks, fine sawdust samples were prepared. OA extraction was performed according to the method proposed by Hunt et al. (2004). Briefly, 100 mg sawdust was placed into a 15 ml centrifuge tube, and 5 ml of 0.1 N NaOH was added. The tube was conditioned at 65°C for 1 h and centrifuged at 21,000×g_0 for 10 min. The supernatant was acidified with 0.25% H_2SO_4 and filtered through a 0.45-µm filter.

Table 1 Retention of CCA in treated blocks[a].

Wood species	Retention (kg m^{-3})			
	CuO	CrO_3	As_2O_5	Total
Radiata pine	0.71±0.02	2.02±0.01	1.27±0.03	4.00±0.02
Western hemlock	0.78±0.03	2.01±0.01	1.22±0.08	4.01±0.21
Japanese cedar	0.77±0.05	2.04±0.21	1.23±0.10	4.04±0.36

[a]Each value represents the mean of three samples.

The OA content in the filtrate was determined by HPLC according to Kim et al. (2009).

Analysis of elements typical for CCA

The same ICP-OES procedure was applied as in case of CCA retention determination in treated wood.

Statistical analysis

Statistical analysis was performed by means of the SAS version 9.1.3 (SAS Institute Incorporated, Cary, NC, USA). Significant differences (α=0.05) in average mass loss (ML), OA production, and percent metal removal were determined by ANOVA and Duncan's multiple range test. Correlation analysis was performed to assess the relationship between ML and OA production. Correlation and regression analyses were also performed to characterise the relationship between the amount of OA produced and element removal rates.

Results and discussion

Biodegradation of CCA-treated wood

Of the seven isolates tested in this study, five brown-rot fungi, *Crustoderma* sp. KUC8065 and KUC8611, *F. palustris* TYP0507 and TYP6137, and *G. sacchari* GYV9595, degraded at least 10% of the original dry weight of *P. radiata* and *T. heterophylla* treated with CCA, but only two isolates of *F. palustris* degraded *C. japonica* treated with CCA (Table 2). Although *A. vaillantii* SEL8501 and the unknown *Polyporales* sp. LAS6497 were confirmed as CCA-tolerant fungi by the "choice test", low degradability of both treated and untreated blocks was observed. Fungal hyphae of *A. vaillantii* SEL8501 and the unknown *Polyporales* sp. LAS6497 fully covered the wood blocks during the decay test, but did not reduce wood mass.

Crustoderma sp. KUC8065 and KUC8611 significantly degraded CCA-treated *P. radiata*, causing a mass loss (ML) of 49%, followed by *F. palustris* TYP0507 and TYP6137, which caused 43.5% ML. CCA-treated *T. heterophylla* and *C. japonica* were significantly degraded by two isolates of *F. palustris* (ML: 20–25%). However, two isolates of *Crustoderma* sp. caused a moderate ML of CCA-treated *T. heterophylla* (20%) and a slight ML of CCA-treated *C. japonica* (6.9% or less). It is reported that *Crustoderma* sp. KUC8065 and KUC8611, which were isolated from CCA-treated *P. radiata* and Japanese red pine, respectively, are strong degraders of CCA-treated *P. radiata*, while their effects were moderate on Douglas fir, *T. heterophylla*, and *C. japonica* (Choi et al. 2009, 2012a). Choi et al. (2012a) reported that *Crustoderma* sp. degraded 60% of

Table 2 Mass loss of treated and control blocks after the 12-week decay test.

Fungal isolate	Mass loss (%)[a]									Temp. (°C)
	Radiata pine			Western hemlock			Japanese cedar			
	Treated		Control	Treated		Control	Treated		Control	
Antrodia vaillantii SEL8501 (EU024959)[b]	0.3±1.2	D[c]	0.1±0.9	C	2.5±0.7	D	0.1±1.3	E	1.0±2.3	D	0.1±1.5	C	25
Crustoderma sp. KUC8065 (AY858355)	49.0±2.8	A	30.6±14.2	B	22.1±3.9	B	37.2±9.1	B	6.9±2.2	C	24.9±12.7	B	30
Crustoderma sp. KUC8611 (EU024960)	46.8±5.8	AB	49.7±14.2	A	20.9±4.2	B	51.9±4.3	A	6.2±3.1	C	26.0±9.1	AB	30
Fomitopsis palustris TYP0507 (EU024964)	43.5±6.9	AB	38.0±2.4	B	23.7±1.6	AB	30.7±3.5	C	24.8±3.3	A	32.5±4.9	A	35
Fomitopsis palustris TYP6137 (JQ410896)	42.3±2.5	B	35.7±2.3	B	26.4±2.4	A	29.9±4.2	C	21.2±4.1	B	30.6±5.5	AB	35
Gyrodontium sacchari GYV9595 (JQ410895)	13.5±4.1	C	32.3±6.9	B	12.1±1.8	C	25.6±2.0	D	5.2±4.8	C	24.9±±2.5	B	30
Unknown *Polyporales* sp. LAS6497 (EU024961)	1.9±1.6	D	1.0±0.7	C	0.2±0.4	D	1.1±3.1	E	0.5±0.4	D	0.5±1.2	C	30

[a]Each value represents the mean of nine samples. [b]GenBank accession number. [c]Mean mass losses with the same letter are not significantly different (statistical significance was $P < 0.05$) using Duncan's multiple range test.

CCA-treated *P. radiata* in the soil-block decay test according to AWPA standard E10-01 (AWPA 2005c). Furthermore, the efficiency of CCA-treated *P. radiata* degradation by the fungus was improved to 68.7% ML by modifying the soil-block test to exclude the feeder strip (Choi et al. 2009). Degradation of treated wood by *Crustoderma* sp. was slightly diminished in this study. Degradation of CCA-treated *P. radiata*, *T. heterophylla*, and *C. japonica* by *F. palustris* was also somewhat lower in this study compared to the results of the soil-block decay test according to AWPA standard E10-01 (Choi et al. 2012a). *Antrodia vaillantii*, which is well known for biodegradation of treated wood (Arango et al. 2009; Choi et al. 2009, 2012a), did not reduce the mass of treated and untreated wood blocks in our study. Although more than 40% of CCA-treated *P. radiata* and more than 20% of CCA-treated *T. heterophylla* and/or *C. japonica* were degraded by *Crustoderma* sp. and *F. palustris*, the assumption was that the addition of nutrients might reduce the ML. However, *G. sacchari* GYV9595, which yielded only slight MLs (2% or less) in the soil-block decay test (AWPA standard E10-01) (Choi et al. 2012a), degraded 13.5%, 12.1%, and 5.2% of CCA-treated *P. radiata*, *T. heterophylla*, and *C. japonica*. Curling et al. (2002) demonstrated that the ability of fungi to degrade wood was significantly affected by the test method. The decay test according to AWPA standard E10-01 based on soil with feeder strips for fungal growth, unlike JIS standard method K 1571. The different efficiencies of CCA-treated wood block degradation by brown-rot fungi was likely due to methodological differences and the presence of added nutrients.

Production of oxalic acid (OA)

The amount of OA produced in treated and untreated wood blocks after the 12-week decay test is presented in Table 3. Two isolates of *F. palustris* produced the highest amount of OA (33.4–43.8 mg g^{-1}) regardless of wood species, followed by the unknown *Polyporales* sp. LAS6497 (26.8–31.9 mg g^{-1}) and *A. vaillantii* SEL8501 (26.4–29.6 mg g^{-1}). The amount of OA produced by these isolates was higher in treated wood blocks than in untreated controls. Clausen and Green (2003) reported that overproduction of OA by Cu-tolerant brown-rot fungi *Postia placenta* (Fr.) M.J. Larsen & Lombard MAD 698, *M. incrassata* TFFH 294, *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb. MD 106R, and *A. vaillantii* FP 90877R, was observed during degradation of treated southern yellow pine with Cu-based wood preservatives including CCA. The authors suggested that the presence of Cu in the preservatives stimulated OA production. *F. palustris* and the unknown *Polyporales* sp. are also Cu-tolerant fungi (Choi et al. 2009; Jang et al. 2012); thus, the overproduction of OA in this study might be due to the Cu component in the treated wood samples.

It is important to note that *A. vaillantii* SEL8501 and an unknown *Polyporales* sp. LAS6497 produced a high amount of OA during the 12-week decay test period (Table 3), although <3% ML occurred (Table 2). *Crustoderma* sp. KUC8611 and *G. sacchari* GYV9595 produced less OA with MLs of 49% and 13.5%, respectively. The development of OA in the course of brown-rot is well documented (Green III et al. 1992; Shimada et al. 1994). However, *A. vaillantii* SEL8501 and an unknown *Polyporales* sp. LAS6497 did not degrade wood blocks despite the high OA production; in other words, there was no strict correlation between OA production and the ability of fungi to degrade treated wood blocks. Micales (1992) drew a similar conclusion and found that various factors, such as enzymes or Fenton oxidative systems, may govern wood degradation. There is evidence that *A. vaillantii* SEL8501 and an unknown *Polyporales* sp. LAS6497 might not secrete extracellular enzymes during

Table 3 Oxalic acid produced in treated and control blocks after the 12-week decay test.

Fungal isolate	Oxalic acid production (mg g^{-1})[a]											
	Radiata pine				Western hemlock				Japanese cedar			
	Treated		Control		Treated		Control		Treated		Control	
Antrodia vaillantii SEL8501	28.9±1.3	B	14.9±1.3	F	26.4±0.4	B	17.1±1.0	A	29.6±1.8	D	17.2±0.8	D
Crustoderma sp. KUC8065	21.3±0.8	C	21.0±0.7	CD	13.6±1.1	D	21.0±0.3	B	10.3±0.2	E	13.5±0.1	E
Crustoderma sp. KUC8611	21.1±0.2	C	21.9±0.4	C	16.6±1.4	C	21.6±0.8	B	10.6±0.6	E	13.7±0.5	E
Fomitopsis palustris TYP0507	42.7±1.8	A	18.4±0.4	E	33.4±0.2	A	16.9±0.9	C	37.4±0.9	B	24.0±1.5	B
Fomitopsis palustris TYP6137	43.8±2.3	A	19.3±0.6	DE	35.4±2.7	A	20.5±0.8	B	41.1±1.0	A	24.0±0.9	B
Gyrodontium sacchari GYV9595	8.6±0.4	D	24.5±1.0	B	10.4±0.6	E	15.5±0.6	D	8.5±0.8	F	19.5±1.1	C
Unknown *Polyporales* sp. LAS6497	29.8±0.5	B	27.2±1.9	A	26.8±0.4	B	26.5±0.7	A	31.9±0.2	C	28.8±0.8	A

[a]Each value represents the mean of three replicates. [b]Mean amounts of oxalic acid produced with the same letter are not significantly different (statistical significance was $P<0.05$) using Duncan's multiple range test.

the 12-week decay test period, and the OA produced by these isolates alone does not degrade wood. Further studies should focus on the relationship between degradation of CCA-treated wood and extracellular enzyme production.

With few exceptions, the amount of OA produced by seven isolates of brown-rot fungi was higher in this study (Table 3) than in the study by Choi et al. (2012a). They evaluated OA production in CCA-treated and untreated wood blocks by the same fungal isolates in soil-block decay tests based on AWPA standard E10-01. In this study, the decay test was conducted according to JIS standard method K 1571, which includes addition of nutrients such as glucose, malt extract and peptone to the culture bottle. It is well known that production of OA by brown-rot fungi is induced by carbon and nitrogen sources. For example, the amount of OA produced by *P. placenta* increased from 11.9 μg ml^{-1} to 270.8 μg ml^{-1} in liquid media with 28 mM glucose, and from 76.5 μg ml^{-1} to 148.68 μg ml^{-1} in media containing 0.5% peptone (Micales 1994).

Although OA production in liquid media is not necessarily translated to woody substrates, it is apparent that nutrient addition improved OA production by the fungal isolates in our study. Production of large amounts of OA during fungal degradation of wood is particularly important in CCA-treated wood, because OA converts Cr and As bound to the cell walls into a water-soluble form that can be extracted from the treated wood (Clausen 2000; Sierra-Alvarez 2007).

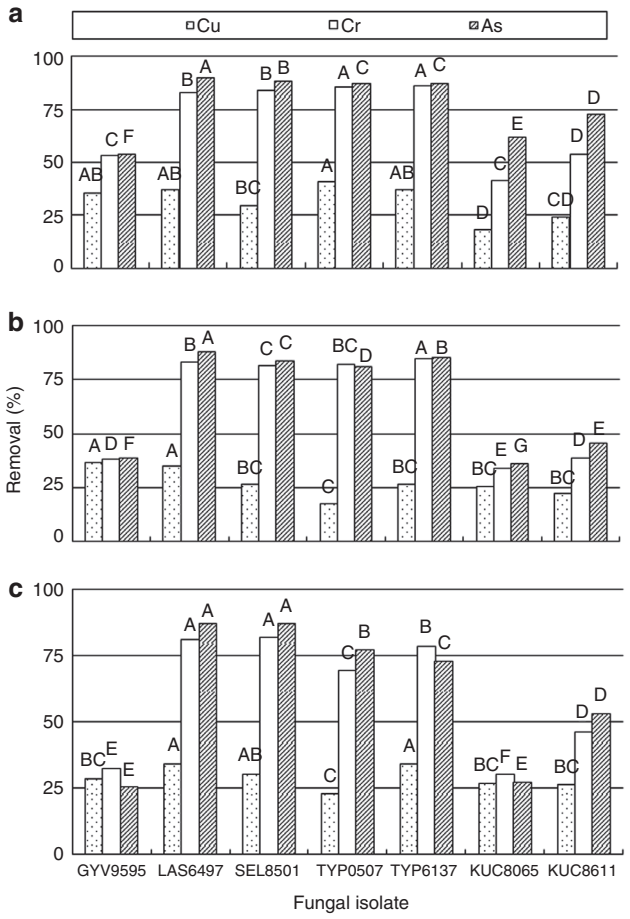

Figure 1 Metal removal rate of CCA-treated radiata pine (a), Western hemlock (b), and Japanese cedar (c).

Removal of CCA elements

Figure 1 shows metal elements removed from the CCA-treated wood blocks during the 12-week decay test. It could be confirmed that Cr and As were more easily removed than Cu from the treated wood, with few exceptions. Of the seven isolates of brown-rot fungi, *A. vaillantii* SEL8501, *Polyporales* sp. LAS6497, and *F. palustris* TYP0507 and TYP6137 yielded remarkable extractability of Cr and As from treated wood blocks.

The unknown *Polyporales* sp. LAS6497 removed the highest amount of As (90.3%) from CCA-treated *P. radiata*, followed by *A. vaillantii* SEL8501 (88.6%), and *F. palustris* TYP0507 and TYP6137 (87.5%) (Figure 1a). The highest removal of Cr from CCA-treated *P. radiata* was produced by two isolates of *F. palustris* (85.5–86%), followed by *A. vaillantii* SEL8501 (83.9%) and unknown *Polyporales* sp. LAS6497 (83.1%). However, only 20–40% of Cu was removed by these fungi from CCA-treated *P. radiata* during 12-week decay test. Large amounts of As (87.6%) and Cr (84.8%) were removed from the treated *T. heterophylla* and *C. japonica* wood blocks, but only a moderate amount of Cu was removed (17.2–34.2%) (Figures 1b and 1c). Bull (2001) demonstrated that extraction of CCA elements with OA has a selective effect on Cr and As because the binding site of Cu differs from those of Cr and As complexes in wood. In high concentrations of OA, Cr or As components can be easily extracted from CCA-treated wood through formation of water-soluble Cr- and As-oxalate, whereas water-insoluble Cu-oxalate is also formed (Choi et al. 2009, 2012a; Kakitani et al. 2009). It was confirmed in this study that *F. palustris* TYP0507 and TYP6137, unknown *Polyporales* sp. LAS6497 and *A. vaillantii* SEL8501, which produced relatively high levels of OA, significantly removed Cr and As from treated wood without significant Cu removal. The extractability of As was slightly higher than that of Cr in most cases, but the difference was minor. The higher extractability of As (Choi et al. 2012b) is due to higher bonding strength of Cr with wood components such as lignin (Pizzi 1990a,b).

The correlation and regression analyses supported the assumed association between OA production and Cr

Table 4 Regression equation for metal removal against oxalic acid production.

Wood species	Cr removal		As removal	
	Equation[a]	R^2	Equation	R^2
Radiata pine	Y=1.619 X+17.826	0.676	Y=1.550 X+21.813	0.654
Western hemlock	Y=2.082 X+11.648	0.782	Y=2.300 X 8.060	0.717
Japanese cedar	Y=62.189 Log X-11.488	0.923	Y=46.712 Log X+16.586	0.881

[a]Y, the amount of Cr or As removed (%); X, the amount of oxalic acid produced (mg g^{-1}).

and As removal (Table 4) and the correlation was statistically significant ($P<0.05$), but there was no significant correlation between OA production and Cu removal. Regression analyses showed logarithmic or linear relationships between OA production and Cr and As removal with high coefficients of determination (R^2=0.654–0.923). As stated above, the OA produced by the brown-rot fungi is sufficient for removal of Cr and As but not for wood degradation. Metal adsorption or uptake by fungal mycelia might also be involved in removal of metals from treated wood (Chou et al. 1973; Choi et al. 2012a), but the reaction mechanisms are not yet clear.

Conclusions

The results show that *F. palustris* degrades CCA-treated wood effectively, causing ML of ca. 43.5%, with extensive extraction of As (87.5%) and Cr (86.0%). *Crustoderma* sp. caused the highest ML of the treated wood, but the isolates only removed moderate amounts of As and Cr. *A. vaillantii* SEL8501 and unknown *Polyporales* sp. LAS6497 caused <3% ML of CCA-treated wood block, but secreted a high amount of OA during the 12-week decay test. The OA produced was significantly involved in the removal of As and Cr from the treated wood, at rates of 90.3% and 83.9%, respectively. It can be stated that *F. palustris* is the most suitable fungus for fungal bioprocessing of CCA-treated wood, causing removal of metals from the treated wood and reduction of the volume and mass of the treated wood simultaneously. *A. vaillantii* and the unknown *Polyporales* sp. are prime candidates for converted CCA-treated wood wastes to feedstock for thermochemical conversion because of the ML they caused.

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