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Bioremediation of CCA-treated wood by brown-rot fungi *Fomitopsis palustris, Coniophora puteana*, and *Laetiporus sulphureus*

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Abstract This study evaluated oxalic acid accumulation and bioremediation of chromated copper arsenate (CCA)-treated wood by three brown-rot fungi *Fomitopsis palustris*, *Coniophora puteana*, and *Laetiporus sulphureus*. The fungi were first cultivated in a fermentation broth to accumulate oxalic acid. Bioremediation of CCA-treated wood was then carried out by leaching of heavy metals with oxalic acid over a 10-day fermentation period. Higher amounts of oxalic acid were produced by *F. palustris* and *L. sulphureus* compared with *C. puteana*. After 10-day fermentation, oxalic acid accumulation reached 4.2 g/l and 3.2 g/l for these fungi, respectively. *Fomitopsis palustris* and *L. sulphureus* exposed to CCA-treated sawdust for 10 days showed a decrease in arsenic of 100% and 85%, respectively; however, *C. puteana* remediation removed only 18% arsenic from CCA-treated sawdust. Likewise, chromium removal in *F. palustris* and *L. sulphureus* remediation processes was higher than those for *C. puteana*. This was attributed to low oxalic acid accumulation. These results suggest that *F. palustris* and *L. sulphureus* remediation processes can remove inorganic metal compounds via oxalic acid production by increasing the acidity of the substrate and increasing the solubility of the metals.

Key words Oxalic acid · Bioremediation · CCA wood preservative · Treated waste wood · *Fomitopsis palustris* · *Coniophora puteana* · *Laetiporus sulphureus*

Introduction

Considerable attention has been focused on the remediation of treated wood in recent years due to public and scientific awareness about the release of chromium, copper, and arsenic from chromated copper arsenate (CCA)-treated waste wood in landfilling, burning, composting, and other modes of disposal. As a result, substantial progress has been made in the remediation of CCA-treated waste wood by chemical extraction with several mineral and organic acids and biodegradation using bacteria and fungi in recent years.[1–11] Previous studies on remediation of CCA-treated wood by extraction with oxalic acid showed that the removal of copper, chromium, and arsenic from CCA-treated waste wood increased significantly.[4,5,7–9,11–13] These studies suggested that oxalic acid plays an important role in partial solubilization of the insoluble metal compounds of CCA wood preservative fixed in the wood. Oxalic acid, the strongest organic acid, has also been implicated directly and indirectly in brown-rot decay processes; however, white-rot fungi fail to accumulate oxalate.[14–16] Milagres et al.[17] also stated that oxalate plays an important role in the degradative attack on cellulose and hemicellulose of wood cells through radical species generated by a Fenton-type reaction. Oxalate is an agent that is small enough to penetrate the cell wall structure of wood and may function in conjunction with metals in the initiation of depolymerization of wood cell components. Oxalate produced by brown-rot fungi is able to complex iron and other metal ions. The properties of these chelators suggest the possibility of utilization in remediation of treated waste wood containing heavy metal ions. Fungi

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have evolved several mechanisms to prevent cellular contact with metals. An extracellular complexation mechanism which prevents cellular contact with metals is provided by the ability of the fungi to produce organic acids such as oxalic acid. The tolerance of some decay fungi to copper has been linked to the amount of oxalic acid produced by the fungi.[18] Preservative-tolerant organisms are of great interest from two different perspectives. Elucidation of the mechanism of tolerance may allow the development of new wood preservatives and the organisms themselves could be used for bioremediation, biodeterioration, and bioconversion of preservative-treated waste wood.[19]

This article reports the detection of oxalic acid produced by the brown-rot fungi *Fomitopsis palustris*, *Coniophora puteana*, and *Laetiporus sulphureus* and bioremediation of CCA-treated wood using liquid cultures of these fungi. In this study, brown-rot fungi were first cultivated and the fermentation medium was then used as a leaching agent to remove heavy metals from CCA-treated wood.

Materials and methods

Wood source

Scots pine (*Pinus sylvestris* L.) poles were obtained from a preservative treatment plant in Adana, Turkey. The poles were previously treated with chromated copper arsenate Type C wood preservative (CCA-C) solution using a full-cell process at a retention of 21 kg/m^3. CCA-C wood preservative contains 18.5% CuO, 47.5% CrO_3, and 34% As_2O_5 as specified by the American Wood Preservers' Association (AWPA) standards.[20]

Sapwood and heartwood portions of the treated poles were separated and the sapwood portions were then chipped with a commercial chipper. The chips were conditioned at 23°C and 65% relative humidity (RH) for 2 weeks.

Analysis of preservative retention in the sawdust

The chips were ground to pass a US Standard 40-mesh screen (420 μm). The sawdust samples (0.1 g) from the poles were then dissolved completely in 10 ml of 65% HNO_3. The sample was transferred into a conical flask equipped with a water cooler to prevent loss by volatilization during the dissolution process. The flask was then heated on a heating plate until the sample had dissolved completely (approximately 4 h). The solution was analyzed for copper, chromium, and arsenic content using an X-ray fluorescence analyzer (XRF) (JSX-3220 JEOL, Nihon Denshi Detamu, Tokyo, Japan) to determine the initial amounts of the elements in the samples.

Microorganisms and cultivation

The fungal strains *Fomitopsis palustris* (Berkeley et Curtis) Murrill (TYP 6137), *Coniophora puteana* (Schum ex Fries) Karsten (COP 6275), and *Laetiporus sulphureus* (Bulliard ex Fries) Bondarcev et Singer (IFO 30745) were obtained from the Laboratory of Biochemical Control, Division of Wood Bioscience, Wood Research Institute, Kyoto University, Japan. Mycelia from established cultures (6–7 days old) incubated on potato/glucose agar (PGA) plates at 28°C were used for the preparation of inocula.

Biomass production

Fungal biomass was cultivated in liquid medium in shaking flasks. The fermentation broth had the following composition: 40 g glucose, 3 g peptone, 15 g malt extract, and 1000 ml deionized water. The broth was autoclaved for 20 min at 121°C. Mycelia from the PGA spreadplate cultures were transferred to 500-ml Erlenmeyer flasks containing 100 ml of fermentation broth. Cultivation of fungi in the flasks inoculated with mycelia was carried out on a rotary shaker at 120 rpm. Culture temperature was maintained at 27°C.

Oxalic acid assay

A known volume of fermentation broth was removed at 1-, 2-, 5-, and 10-day intervals from the flasks inoculated with the fungi. These samples were centrifuged at 10000 rpm and 4°C for 10 min to remove mycelium using a high speed refrigerated microcentrifuge (Tomy MRX 152, Seiko, Tokyo, Japan). The cell-free supernatants were kept in a freezer before being analyzed for oxalic acid.

The amount of oxalic acid formed during fermentation was enzymatically determined using an oxalate kit (Roche R-Biopharm, Darmstadt, Germany). In this assay, oxalate is converted into carbon dioxide and formate (formic acid) at pH 5.0 in the presence of oxalate decarboxylase. The formate formed is quantitatively oxidized to bicarbonate by nicotinamide-adenine dinucleotide (NAD^+) at pH 7.5 in the presence of the enzyme formate dehydrogenase. At the end of the reaction, NAD^+ is reduced to NADH (the reduced form of NAD^+). The amount of NADH formed during the reaction is stoichiometric to the amount of oxalate and is determined spectrophotometrically at 340 nm using a double-beam spectrophotometer (Hitachi U-3000, Hitachi, Tokyo, Japan) equipped with a temperature controller and external recorder.

Bioremediation of CCA-treated wood

CCA-treated sawdust was placed into teabags made from polyester fibers. The teabags containing sawdust were sterilized with gaseous ethylene oxide before bioremediation. Each bag containing sawdust (3 g) was placed in flasks containing 100 ml of fermentation broth inoculated with *F. palustris*, *C. puteana*, or *L. sulphureus* for 10 days. The flasks were agitated for 1, 2, 5, and 10 days at 120 rpm on a rotary shaker at 27°C. Uninoculated fermentation broth (UFB) and deionized (DI) water extraction served as a control. Two bags of sawdust were removed at each time interval

and rinsed three times with 300 ml of DI water at 20°C. Bioremediated sawdust was oven-dried at 60°C for 24 h and conditioned at 23°C and 65% RH for 2 weeks. The bioremediated sawdust was then analyzed for remaining copper, chromium, and arsenic content using an XRF analyzer as described above. The percent reduction of copper, chromium, and arsenic in the sawdust samples was calculated based on the initial amounts of the elements in the samples.

Results and discussion

Oxalic acid production

Oxalic acid was the only organic acid detected in the fermentation broths in this study. Figures 1, 2, and 3 show oxalic acid concentration and pH in the broth during remediation by *Fomitopsis palustris*, *Coniophora puteana*, and *Laetiporus sulphureus*, respectively. The accumulation of oxalic acid and pH of the broth were followed as a function of time. *Fomitopsis palustris* demonstrated the highest levels of oxalic acid accumulation per liter of fermentation broth and the highest pH reduction. After 2 days of fermentation, substantial amounts of oxalic acid accumulated in the fermentation broth of *F. palustris* (3.62 g/l). Being dependent on accumulated oxalic acid, the pH of the broth decreased from 5.6 to 3.6. In contrast, the fermentation broth of *C. puteana* showed the lowest oxalic acid accumulation, although this fungus also caused a lowering of fermentation broth pH. This is probably related to other organic acids produced during fermentation. Even though *F. palustris* produced the highest amount of oxalic acid at the end of the fermentation, oxalic acid content and pH during fermentation were almost constant after 5 days. However, *L. sulphureus* showed increasing oxalic acid concentration and decreasing pH during the entire fermentation duration. Compared to *F. palustris*, *L. sulphureus* did not show fast pH decrease and oxalic acid accumulation during the fermentation period.

Exposure of CCA-treated wood to highly acidic conditions may reverse the fixation process in which insoluble CCA components form complexes with wood cell components.[5] Oxalic acid, a chelating and reducing agent, is one of the strongest organic acids (pKa_1 1.19). Oxalic acid has been implicated directly and indirectly in the brown-rot decay process in wood and is also involved in pH reduction and

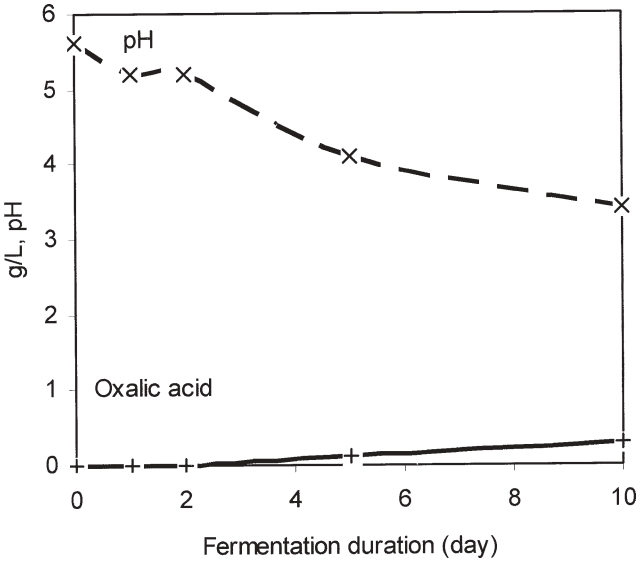

Fig. 2. Oxalic acid concentration and pH during *Coniophora puteana* fermentation

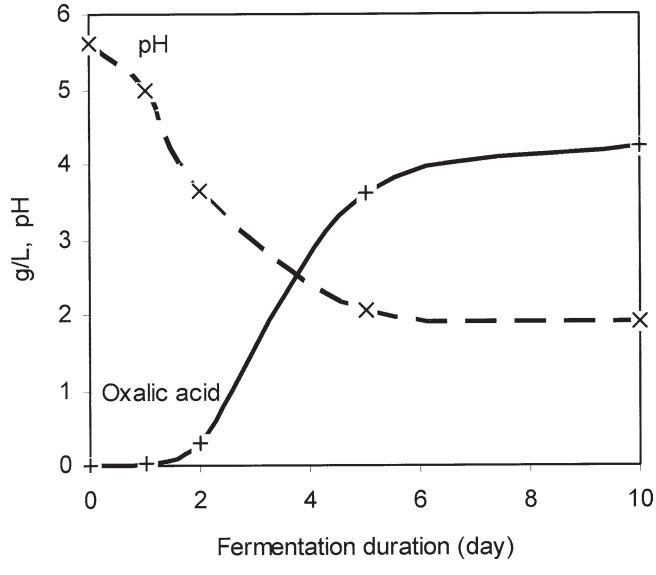

Fig. 1. Oxalic acid concentration and pH during *Fomitopsis palustris* fermentation

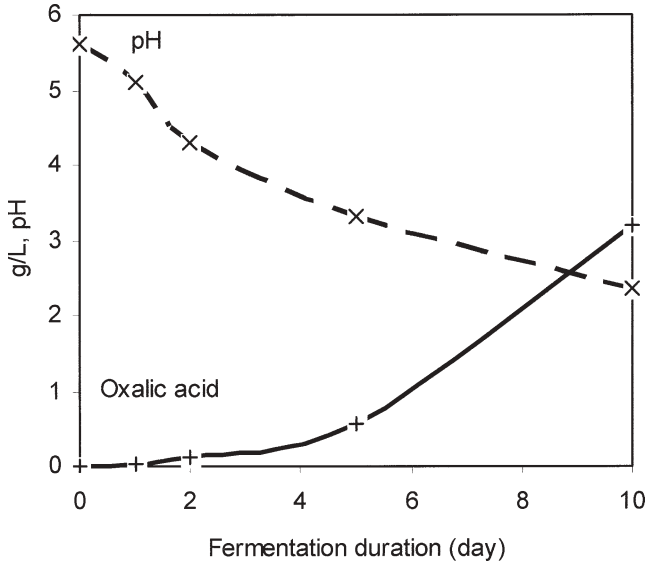

Fig. 3. Oxalic acid concentration and pH during *Laetiporus sulphureus* fermentation

acid-catalyzed hydrolysis of the wood substrate.[14,15] The ability of some fungal strains to accumulate oxalic acid indicates that there may be alternatives to commercial oxalic acid for the remediation of CCA-treated waste wood. In addition to the strong acidic character of oxalic acid, it is the dominant organic acid produced by brown-rot fungi.[14] However, oxalic acid accumulation by wood-decaying fungi shows variation between species. For instance, in brown-rot fungi, secretion of oxalic acid continues throughout the life cycle, while white-rot fungi secrete oxalic acid as a secondary metabolism product.[21] In the present study, *F. palustris*, *C. puteana*, and *L. sulphureus* produced 4.2 g/l, 0.3 g/l, and 3.2 g/l oxalic acid, respectively, after a 10-day fermentation. Green and Clausen[22] found that two species of *Fomitopsis*, including *palustris*, and *L. sulphureus* showed the highest concentration of oxalic acid accumulation (4.7 and 3.9 μM/mg mycelium, respectively). On the other hand, they reported that *C. puteana* did not show high oxalic acid accumulation (0.7 μM/mg mycelium). The oxalic acid produced by wood-degrading fungi reduces the pH and creates a pH gradient.[23] In the present study, as oxalic acid accumulation increased, the pH of the fermentation broth decreased simultaneously. However, *C. puteana* caused a decreased pH in the fermentation broth despite low oxalic acid accumulation in the broth. This indicates that other organic acids may be produced during fermentation.

Removal of copper, chromium, and arsenic

Results of X-ray fluorescence spectroscopy analysis for CuO, CrO_3, and As_2O_5 following remediation by fungi used in the study of CCA-treated sawdust are shown in Table 1. Results are expressed as mg of each component remaining per gram of treated wood following remediation. Values represent the average of duplicate samples. As bioremediation duration increased, remaining CCA components in sawdust decreased gradually. CCA-treated sawdust contained 11.2 mg/g CuO, 26.3 mg/g CrO_3, and 17.2 mg/g As_2O_5. However in the sawdust exposed to *F. palustris*, all As_2O_5 was removed after 10-day remediation. Compared to *C. puteana*, *F. palustris* and *L. sulphureus* caused more CCA component removal during remediation depending on the amount of oxalic acid accumulation before bioremediation; however, CuO removal in *C. puteana* was greater than that in the *L. sulphureus* remediation process.

The percentages of copper, chromium, and arsenic elements removed from CCA-treated sawdust exposed to deionized (DI) water, uninoculated fermentation broth (UFB), *F. palustris*, *C. puteana*, and *L. sulphureus* remediation processes at varying durations are shown Figs. 4, 5, 6, 7, and 8, respectively. The total percentage of elements removed from sawdust treated with DI water was lower than those for samples treated with UFB and fungal fermentation. Extraction with DI water for 10 days removed about 13% copper, 11% chromium, and 14% arsenic from CCA-treated sawdust. Exposure of treated sawdust to UFB for 10 days enhanced the removal of metals. This remediation process removed about 39%, 16%, and 19% of the initial concentrations of copper, chromium and arsenic, respectively, in CCA-treated sawdust. These results suggested that the UFB, containing mainly glucose, had a potential to absorb metals released from CCA-treated sawdust.[24] This suggests that UFB is able to remove copper

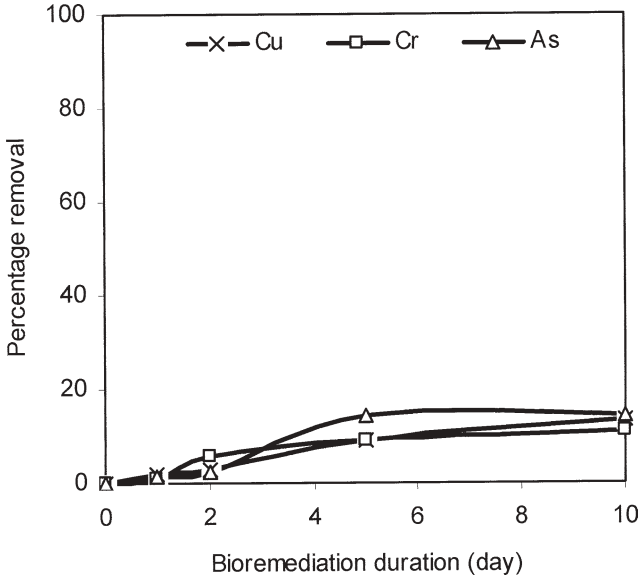

Fig. 4. Percentage removal of elements from CCA-treated sawdust by deionized (DI) water extraction

Table 1. Amount of chromated copper arsenate (CCA) components remaining in sawdust samples following exposure to deionized water, uninoculated fermentation broth (UFB), and *Fomitopsis palustris*, *Coniophora puteana*, and *Laetiporus sulphureus* fermentation (mg/g)[a]

Bioremediation duration (day)	Exposure conditions														
	Deionized water			UFB			*F. palustris*			*C. puteana*			*L. sulphureus*		
	CuO	CrO_3	As_2O_5	CuO	CrO_3	As_2O_5	CuO	CrO_3	As_2O_5	CuO	CrO_3	As_2O_5	CuO	CrO_3	As_2O_5
1	10.91	25.96	16.96	7.31	23.44	15.33	5.89	15.94	7.61	4.58	21.68	13.70	7.51	8.54	3.56
2	10.81	24.75	16.82	6.65	22.21	14.29	4.66	7.87	2.26	4.01	21.98	14.52	6.56	8.51	2.81
5	10.15	23.90	14.79	7.03	23.19	14.07	2.86	6.57	1.01	3.96	21.79	14.18	6.44	8.47	2.81
10	9.69	23.44	14.74	6.76	22.08	14.02	3.13	3.36	0.00	3.73	21.29	14.12	5.56	8.14	2.59

CCA-treated wood contained 11.15 mg/g CuO, 26.27 mg/g CrO_3, and 17.22 mg/g As_2O_5 before remediation
[a] Values represent the average of duplicate samples

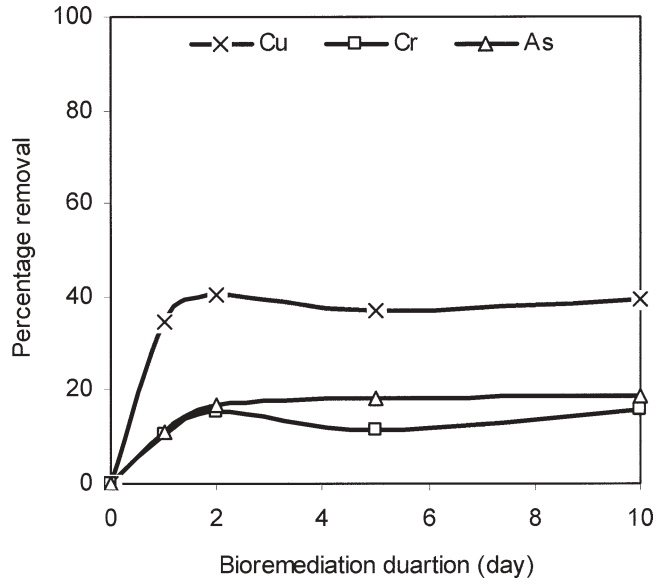

Fig. 5. Percentage removal of elements from CCA-treated sawdust by uninoculated fermentation broth (UFB) extraction

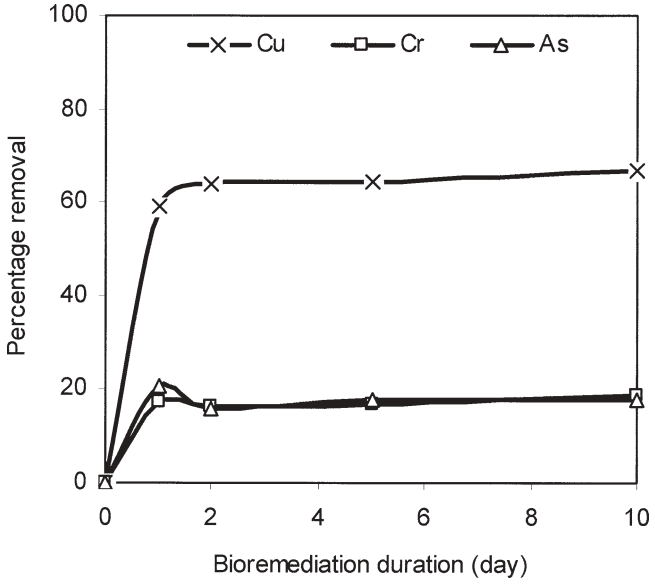

Fig. 7. Percentage removal of elements from CCA-treated sawdust by *C. puteana* fermentation

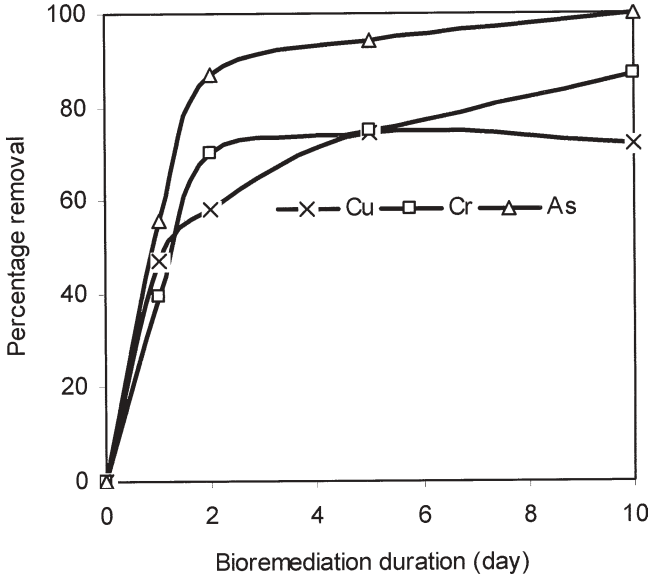

Fig. 6. Percentage removal of elements from CCA-treated sawdust by *F. palustris* fermentation

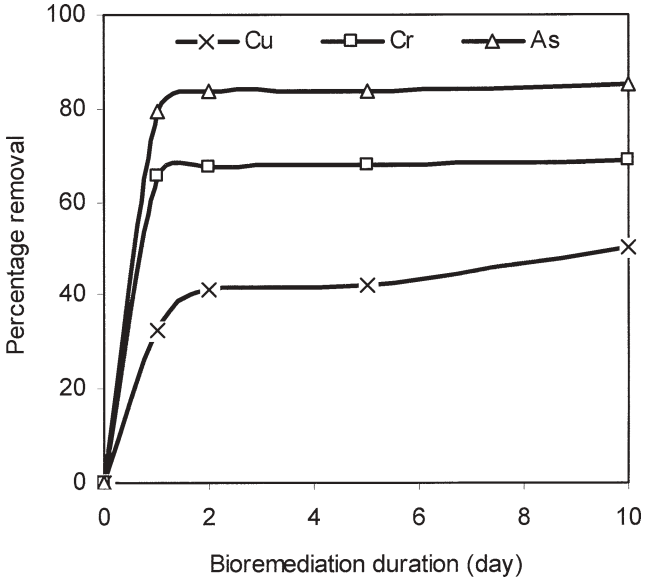

Fig. 8. Percentage removal of elements from CCA-treated sawdust by *L. sulphureus* fermentation

and arsenic as effectively as *C. puteana*. However, total percentages of copper, chromium, and arsenic removed from treated sawdust following *F. palustris* and *L. sulphureus* remediation processes were considerably higher than extractions with DI water and UFB. Percentage metal removal followed the order *F. palustris* > *L. sulphureus* > *C. puteana*. *Fomitopsis palustris* remediation of CCA-treated sawdust for 10 days removed about 72% copper, 87% chromium, and 100% arsenic while 50% copper, 69% chromium, and 85% arsenic were removed from treated sawdust after a 10-day *L. sulphureus* remediation. On the other hand, the lower oxalic acid accumulation of *C. puteana* caused less chromium and arsenic removal after a 10-day remediation. The reductions of copper, chromium, and arsenic by *C. puteana* remediation were about 67%, 19%, and 18%, respectively. Figure 9 shows the percentage of copper, chromium, and arsenic removed from treated sawdust following DI water and UFB extraction, and *F. palustris*, *C. puteana*, and *L. sulphureus* remediation for 10 days.

Previous studies[2,5,7–9,12,13] have shown that the removal of copper, chromium, and arsenic from CCA-treated wood waste increased significantly during oxalic acid extraction because oxalic acid functions as a chelating agent to sequester metal ions and reduces the pH to provide acid conditions for remediation. In a study by Kartal et al.,[24] oxalic

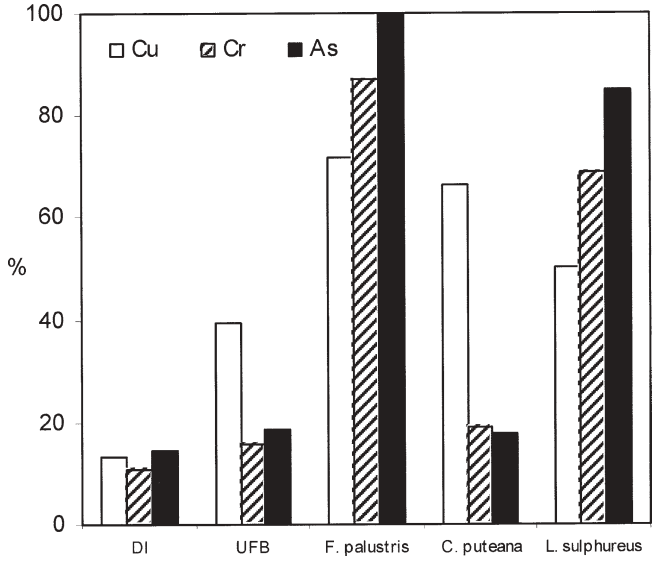

Fig. 9. Percentage of copper, chromium, and arsenic released following deionized (*DI*) water and uninoculated fermentation broth (*UFB*) extraction, *F. palustris*, *C. puteana*, and *L. sulphureus* fermentation for 10 days

acid production by *Aspergillus niger*, an Ascomycetes fungus, was 13.4 g/l at pH 6 in an enriched nitrogen and phosphorus medium. *Aspergillus niger* exposed to CCA-treated chips for 10 days showed a decrease in arsenic of 97%. In addition, *A. niger* fermentation removed 49% copper and 55% chromium from CCA-treated chips. Our study showed that arsenic was the element most affected during remediation by *F. palustris* and *L. sulphureus*. Compared with arsenic, less copper and chromium were removed by remediation with these fungi. However, *C. puteana* remediation caused more copper removal compared with chromium and arsenic removal despite its low oxalic acid accumulation during the fermentation. *Coniophora puteana* remediation also removed more copper from CCA-treated sawdust than *L. sulphureus* remediation. This might be explained by the low starting pH of the medium governing metal sorption.[17,23]

Conclusions

Some fungal strains are able to produce a quite high concentration of oxalic acid. Oxalic acid, a chelating and reducing agent, is one of the strongest organic acids. Because it is readily oxidized, it is also useful as a reducing agent for bleaching and ink removal. This study evaluated the removal of copper, chromium, and arsenic from CCA-treated wood using *Fomitopsis palustris*, *Coniophora puteana*, and *Laetiporus sulphureus* remediation. Oxalic acid produced by the fungi during fermentation was used for the removal of metal elements via bioleaching. *Fomitopsis palustris* and *L. sulphureus* remediation removed 100% and 85% of arsenic found in the CCA-treated wood, respectively. Compared with arsenic, less copper and chromium were removed by remediation with these fungi. We also observed that uninoculated fermentation broth containing mainly glucose can be used as a biosorbent for the removal of copper. Fungi that are able to produce high amounts of oxalic acid can be considered as a possible alternative to commercial oxalic acid extraction for the acid extraction of treated wood.

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