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# Influence of corn steep liquor and glucose on colonization of control and CCB (Cu/Cr/B)-treated wood by brown rot fungi

Miha Humar [a,*], Sam A. Amartey [b], Franc Pohleven [a]

[a] University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Rozna dolina, c. VIII/34, SI-1000 Ljubljana, Slovenia
[b] Forest Products Research Center, Buckinghamshire Chilterns, University College, High Wycombe HP11 2JZ, UK

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**Abstract**

There are increasing problems with regard to the disposal of treated wood waste. Due to heavy metals or arsenic in impregnated wood waste, burning and landfill disposal options are not considered to be environmentally friendly solutions for dealing with this problem. Extraction of the heavy metals and recycling of the preservatives from the wood waste is a much more promising and environmentally friendly solution. In order to study the scale up of this process, copper/chromium/boron-treated wood specimens were exposed to copper tolerant (*Antrodia vaillantii* and *Leucogyrophana pinastri*) and copper sensitive wood decay fungi (*Gloeophyllum trabeum* and *Poria monticola*). Afterwards, the ability of fungal hyphae to penetrate and overgrow the wood specimens was investigated. The fungal growths were stimulated by immersing the specimens into aqueous solution of glucose or corn steep liquor prior to exposure to the fungi. The fastest colonization of the impregnated wood was by the copper tolerant *A. vaillantii*. Addition of glucose onto the surface of the wood specimens increased the fungi colonization of the specimens; however, immersion of the specimens into the solution of corn steep liquor did not have the same positive influence. These results are important in elucidating copper toxicity in wood decay fungi and for using these fungi for bioremediation of treated wood wastes.


## 1. Introduction

Aqueous solutions of chromated copper arsenate (CCA) and of chromated copper borate (CCB) are the most important wood preservatives, as CCA and CCB-treated lumber have been proven to be effective in deterring insects and fungal decay. For example, approximately 212 million $m^3$ of CCA-treated lumber was produced annually in the USA (Clausen and Smith, 1998). Additionally, 1.5–2.0 million $m^3$ of wood containing about 1000 tons of chromium and 600 tons of copper are preserved annually in the area of the former Federal Republic of Germany (Stephan et al., 1996). Therefore, it is anticipated that huge amounts of preserved wood would be removed from service in many developed countries in future years. The presence of copper, chromium, and particularly arsenic could cause problems in the disposal of this impregnated wood, due to the toxic nature of these elements. Thus, it is very important to find an effective and environmentally sound recycling solution for this preserved wood when removed from service.

Landfill disposal is not an environmentally sound option for treated wood waste, since it only postpones dealing with the problem to future generations. Furthermore, the heavy metals in the wood may diffuse into the surrounding soil, resulting in significant environmental damage (Stephan and Peek, 1992; Clausen and Smith, 1998). In addition, capacities of special landfill sites for the disposal of these impregnated wood wastes are limited and public approval for new facilities is extremely low. Burning of CCA/CCB preserved wood waste is only permitted in approved incinerators under closely controlled conditions in many countries, since emitted

* Corresponding author. Tel.: +386 1 423 1161; fax: +386 1 423 5035.
*E-mail address:* miha.humar@bf.uni-lj.si (M. Humar).

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gases have been found to contain high concentrations of arsenic compounds (Honda et al., 1991). The cost of destruction, such as by incineration, has also been found to be very expensive (Ribeiro et al., 2000).

A number of environmentally sound disposal options have been investigated in recent years, including biological methods using either copper tolerant fungal strains (Stephan et al., 1996) or bacteria (Clausen and Smith, 1998). The principle underlying these methods is to convert the insoluble heavy metals in the waste wood into a soluble form through acidification with organic acids. The soluble heavy metal complex can then be leached from the wood. Thus, both the remediated wood fiber and the metals can be reclaimed and recycled. If managed properly, the detoxified spent wood can be a good source of fiber for the manufacturing of engineering products such as fiberboard, particleboard and wood cement composites or it can also be hydrolyzed to simple sugars, which can be converted through fermentation to products such as ethanol, organic acids (lactic, acetic and formic) and xylitol. The recovered metals can be recycled to produce wood preservatives. The most important acid involved in this process is oxalic acid (Clausen and Smith, 1998), a small organic acid with two low p$K$ values (p$K_1$ = 1.27; p$K_2$ = 4.26) (Skoog et al., 1992), in great quantities produced by brown rot fungi (Green III et al., 1991; Humar et al., 2001, 2002) and associated with brown rot colonization of wood (Jellison et al., 1997). The most efficient oxalic acid producers and consequently the most tolerant fungi include the genus *Antrodia* (Tsunoda et al., 1997). Other wood decaying fungi produce significantly less oxalic acid. Instead of oxalic acid, these fungi excrete other organic acids in order to optimise the pH value of the substrate (Takao, 1965; Humar et al., 2001). Oxalic acid can react with insoluble chromium in wood to form chromium oxalate, which is soluble and can be leached out of the wood. On the other hand, copper oxalate, which is formed between copper and oxalic acid, is insoluble and can only be leached with an ammonia solution (Stephan and Peek, 1992; Humar et al., 2004).

The aim of this study was to elucidate the ability of a selected copper tolerant fungal strain as well as copper sensitive ones to colonize wood treated with copper and chromium based preservatives and to determine the influence of nutrient sources (glucose and/or corn steep liquor (CSL)) for improving colonization of the wood. We suspect that the nutrients would stimulate initial fungal growth and colonization of the wood. In this experiment, a relatively inexpensive and readily available nutrient source, CSL, was used. It is a waste product from the wet corn milling process when the dry corn is soaked in a warm dilute sulfurous acid solution. During the process, the grain solubles are released and undergo mild lactic acid fermentation from naturally occurring bacteria. CSL contains high amounts of nitrogen; therefore, it was used in several biotechnological processes (Akhtar et al., 1997). For comparison, pure glucose was used. Although it is easily available, it is not a cheap and economically viable carbon source.

## 2. Materials and methods

### 2.1. Preparation of the samples

Norway spruce (*Picea abies*) samples (15 × 25 × 50 mm) were vacuum impregnated with 5% CCB solution according to the EN 113 procedure (ECS, 1989). The treatment resulted in a preservative uptake of about 16 kg/m$^3$. The samples were later conditioned for 4 weeks, the first 2 weeks in closed chambers, the third week in half closed chambers and the fourth week in open chambers. The conditioned samples were then oven-dried (75 °C) for 5 d in order to ensure complete reduction of chromium. Following conditioning, the samples were leached according to the EN 84 procedure for 14 d (ECS, 1994). Afterwards, the samples were oven-dried (103 °C) and their masses were determined, then conditioned and finally steam-sterilized. Prior to exposure to the fungi, some samples were immersed for 5 min in a 4% aqueous solution of corn steep liquor (CSL) (Sigma), 4% aqueous solution of glucose, a mixture of 4% aqueous solution of CSL and glucose (Sigma, or in distilled water only). Glucose and CSL were used as easy available carbon and nitrogen sources, respectively.

### 2.2. Baiting experiment

The experiment was performed according to the procedure described by Kleist et al. (2002). This experiment was designed to determine whether fungal hyphae can penetrate the center of the wood sample or can only be found in the surface region. Samples impregnated as described above were prepared as follows prior to exposure to the fungi. Holes (diameter = 3 mm, depth = 20 mm) were bored in a longitudinal direction into the center of the sample. A small toothpick was then inserted into the hole as the bait and the hole sealed with epoxy sealer, as shown in Fig. 1. The epoxy sealer has no effect on fungal growth. Afterwards, the samples were

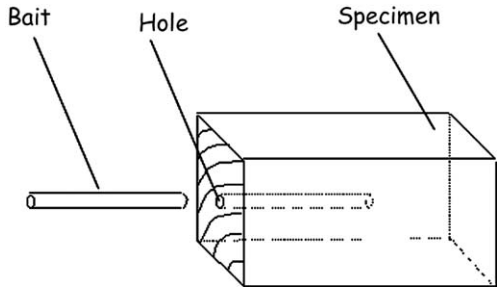


Fig. 1. Sketch of the specimen used for the baiting experiment.

sterilized and exposed to the fungi as described later. After 1, 2, or 4 weeks of exposure, the toothpicks were carefully removed from the specimens under sterile conditions and put onto a sterilized solid nutrient medium (PDA, Difco). Any fungal growth from the sticks on the nutrient medium was monitored for a period of 2 weeks.

### 2.3. Exposure to the fungus

Sterilized and air-dried samples were exposed to the brown rot fungi listed in Table 1. The *Antrodia vaillantii* and *Leucogyrophana pinastri* strains have been shown to be copper tolerant in previous investigations (Humar et al., 2001; Pohleven et al., 2002). Cultures were grown and maintained on a 3.9% potato dextrose agar medium (PDA, Difco). Jars with PDA medium were inoculated with small pieces of fungal mycelium. One treated and one untreated wood sample were placed on a sterilized plastic grid in each inoculated jar and exposed to fungal decay for certain period of time in the growth chamber (25 °C, RH = 75%).

### 2.4. Respiration measurements

Infested wood blocks were, after 5 weeks of exposure, carefully removed from the fungal mycelia and put into empty experimental jars where they were subjected to measurement of $CO_2$ production. The jars were sealed with ventilation lids, using silicon vacuum paste to enhance the seal. Afterwards the initial concentration of $CO_2$ and the final concentration after 1 h were measured using an equipment that consisted of a membrane pump (flow rate 0.5 l/min), IR carbon dioxide sensor (0–3000 ppm, accuracy = 5 ppm) and 16-bit A/D converter for computerized data acquisition (ECHO d.o.o. Slovenia). The ECHO system was a closed-circuit system, which permitted measurements of changes in $CO_2$ concentrations over time (Tavzes et al., 2002).

## 3. Results and discussion

### 3.1. Colonization of the control specimens

The fastest overgrowth of the control-unimpregnated specimens was by *Gloeophyllum trabeum* followed by *Poria monticola*. Hyphae of *G. trabeum* reached the bait in the center of specimens after 1 week of exposure for two-thirds of the exposed specimens. However, specimens exposed to the other brown rot fungi investigated apart from *L. pinastri* were completely colonized after 2 weeks of exposure. *L. pinastri* took more time to achieve 100% colonization of the specimens (Table 2).

This result correlates well with the respiration measurements of the infested wood specimens. *G. trabeum* and *P. monticola*, which showed the highest ability to penetrate the wood specimens, produced the highest levels of $CO_2$ after 4 weeks of exposure. The copper sensitive *G. trabeum* produced, on average, 1127 ppm of $CO_2$ in 1 h, which was almost double the amount produced by the copper tolerant *A. vaillantii* (560 ppm of $CO_2$/h). These results are comparable with the respiration rates of these fungi described by Tavzes et al. (2002) (Fig. 2). Intense production of $CO_2$ reflected in higher mass losses obtained as well. This can be clearly seen from a comparison of *G. trabeum* with *A. vaillantii*. After 5

Table 1
Brown rot fungi used

| Fungi | Abbreviation | Origin | Estimated Cu tolerance |
|---|---|---|---|
| *Antrodia vaillantii* | Av | University of Ljubljana, ZIM L037* | Cu tolerant 1 |
| *Leucogyrophana pinastri* | Lp | Buckinghamshire Chilterns University College | Cu tolerant 2 |
| *Poria monticola* | Pm | BAM 102, Germany | Cu sensitive/Cu tolerant 3 |
| *Gloeophyllum trabeum* | Gt | University of Ljubljana, ZIM L017* | Cu sensitive 5 |

Copper tolerance is described with marks according to the results of Pohleven et al. (2002). Mark 1 describes the highest copper tolerance; and 5 describes the highest copper sensitivity.
(Raspor et al., 1995).

Table 2
Percentages of colonized untreated specimens exposed to the fungi for 1, 2 and 5 weeks

| Fungus | Percentages of colonized specimens (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 week | | | | 2 weeks | | | | 5 weeks | | | |
| | C | G | CSL | CSL + G | C | G | CSL | CSL + G | C | G | CSL | CSL + G |
| Av | 0 | 33 | 0 | 33 | 100 | 100 | 75 | 75 | 100 | 100 | 100 | 100 |
| Lp | 0 | 0 | 0 | 0 | 100 | 100 | 75 | 75 | 100 | 100 | 100 | 100 |
| Pm | 33 | 33 | 0 | 33 | 100 | 100 | 75 | 100 | 100 | 100 | 100 | 100 |
| Gt | 66 | 66 | 0 | 0 | 100 | 100 | 75 | 75 | 100 | 100 | 75 | 100 |

Prior to the fungal exposure, samples were immersed into water (C), aqueous solution of glucose (G), corn steep liquor (CSL), or glucose and corn steep liquor (CSL + G).

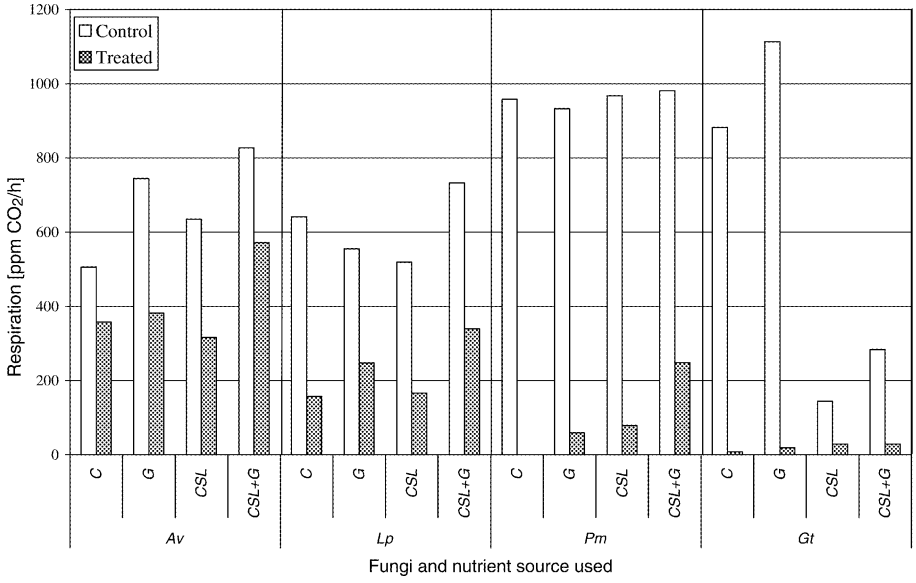


Fig. 2. Respiration of infested unimpregnated wood samples (control) and impregnated (treated) and specimens exposed to the fungi for 5 weeks. Prior to the fungal exposure, samples were immersed in water (C), aqueous solution of glucose (G), corn steep liquor (CSL), or glucose and corn steep liquor (CSL + G).

Table 3
Mass losses of the impregnated and control specimens exposed to the brown rot fungal strains for five weeks

| Fungus | Nutrient source | Mass loss (%) | |
|---|---|---|---|
| | | Unimpregnated specimens | Impregnated specimens |
| Av | CSL | 2.8 | 0.6 |
| | CSL + G | 6.7 | 0.9 |
| | G | 4.9 | 2.4 |
| | Control | 4.2 | 1.2 |
| Lp | CSL | 1.9 | 0.3 |
| | CSL + G | 3.1 | 0.6 |
| | G | 3.6 | 0.6 |
| | Control | 2.6 | 0.2 |
| Pm | CSL | 12.5 | 0.4 |
| | CSL + G | 14.2 | 0.7 |
| | G | 11.7 | 0.5 |
| | Control | 9.1 | 0.3 |
| Gt | CSL | 1.1 | 0.3 |
| | CSL + G | 1.7 | 0.7 |
| | G | 11.1 | 0.4 |
| | Control | 10.1 | 0.2 |

Prior to the fungal exposure, samples were immersed in water (control), aqueous solution of glucose (G), corn steep liquor (CSL), or glucose and corn steep liquor (CSL + G).

weeks, *G. trabeum* decayed 10.1% of the control specimens, what was approximately 2.5 times more than the copper tolerant *A. vaillantii* (4.2%) (Table 3).

The addition of nutrients did not increase the ability of fungi to reach the centers of the unimpregnated samples. However, in the presence of CSL and/or glucose, very few fungal hyphae reached the bait in the samples (Table 2). The copper tolerant strain *A. vaillantii* colonized all control specimens that were immersed in only water prior to exposure in 2 weeks. On the other hand, only 75% of samples that were immersed in aqueous solution of CSL or a mixture of CSL and glucose were completely colonized after a 2-week period. A similar relationship was observed for the other fungal species as well. Colonization of the specimens that were immersed in the glucose solution, were comparable or even faster than colonization of the control specimens (Fig. 2). This result was further supported by the results of the respiration measurements. We believe that this could be due to the fact that brown rot fungi generally needs less nitrogen for growth compared to white rot fungi (Carlile and Watkinson, 1994). Therefore, the presence of CSL might even inhibit fungal growth. However, CSL as energy source in biotechnological processes was first used for bio-pulping, with white rot fungi (Akhtar et al., 1997). This indicates that the same nutrient source cannot be used for both white and brown rot fungi. The performance of CSL has to be studied and optimized for each bioprocess.

Respiration measurements indicate more clearly than the baiting experiment, that while in some cases the addition of nutrients has a positive influence on fungal growth (glucose), in certain cases, the nutrients were of no influence (Pm) and in other cases, the nutrients had a negative influence (CSL) on growth of the wood decay fungi on the wood surface (Fig. 2). In general, unimpregnated samples that were immersed in glucose were much more overgrown by the fungi than the other samples. For example, *A. vaillantii* growing on unimpregnated specimens immersed in glucose produced 744 ppm $CO_2$/h, while growth on specimens immersed in water produced 505 ppm $CO_2$/h. This result was expected, as glucose is a readily available nutrient source, which can easily be

consumed by fungi without expending energy. In contrast, CSL has significantly less positive influence on the growth of the fungi compared to glucose. This can be clearly seen from the unimpregnated specimens exposed to *G. trabeum*. Results (Fig. 2) show that there was significantly less growth on the surface of the specimen. The production of carbon dioxide by *G. trabeum* while overgrowing samples immersed in CSL was more than six times lower than the ones immersed in water only (Fig. 2). The observed $CO_2$ level also reflects the mass loss obtained. *G. trabeum* decayed 10.1% of control specimens, and 11.1% of specimens that were immersed in only glucose in 5 weeks. On the other hand, significantly lower mass losses of specimens immersed in CSL (1.1%) and CSL/glucose (1.7%) were obtained (Table 3). Similar ratios were observed for copper tolerant *L. pinastri* as well, which further demonstrates the negative influence of CSL on the growth and decay abilities of some brown rot fungal strains.

### 3.2. Colonization of the impregnated specimens

Colonization of the impregnated specimens was significantly lower compared to the control ones. Not all of the fungal isolates were able to penetrate the center of the impregnated specimens after 2 weeks of exposure. After 5 weeks of exposure, the first hyphae of *A. vaillantii* reached the bait in the center of the treated specimens. However, complete colonization was observed after 7 weeks of exposure. Colonization of the impregnated specimens by the copper tolerant *A. vaillantii* was not surprising, as this strain was found to exhibit high copper tolerance (Pohleven et al., 2002; Humar et al., 2004). First hyphae of the other copper tolerant fungi *L. pinastri* reached the bait in the center of the impregnated samples after 8 weeks of exposure and complete (100%) colonization was observed after 12 weeks. However, none of the copper sensitive fungal species reached the center of the impregnated specimens even after 12 weeks of exposure.

Respiration measurements were performed after 5 weeks of exposure of the impregnated specimens to the fungi. Higher overgrowth of the control specimens compared to the impregnated ones was evident visually as well as from the $CO_2$ measurements. The highest surface overgrowth of the treated specimens was found on the CCB impregnated specimens exposed to the copper tolerant *A. vaillantii* followed by *L. pinastri*. Production of $CO_2$ from the leached impregnated specimens exposed to *A. vaillantii* was approximately 66% of that from the control samples. On the other hand, exposure of the impregnated samples to the copper sensitive species *G. trabeum* resulted in 100 times lower $CO_2$ production than the control ones. However, both visual observation and $CO_2$ measurement showed insignificant growth on the impregnated wood exposed to the copper sensitive *P. monticola* (Fig. 2).

As observed with the un-impregnated samples, immersion of the impregnated specimens in CSL and/or glucose did not significantly influence growth of the fungi to the center of the impregnated specimens (Table 4). On the other hand, immersion in nutrients significantly improved growth of the fungi on the surface of the impregnated specimens (Fig. 2). This was much more evident with the copper sensitive fungal strain. There was no growth observed on specimens immersed in only water and exposed to Pm. In contrast, when the specimens were immersed in CSL and glucose, 247 ppm of $CO_2$/h was produced. The highest respiration rates were observed with impregnated specimens immersed in aqueous solution of CSL and glucose prior to exposure (Fig. 2). Similar correlation was also found for the mass losses of the specimens exposed to copper tolerant *A. vaillantii*. This fungal strain decayed 1.2% of the CCB impregnated specimen, 2.4% of the impregnated specimen immersed in the glucose solution and 0.6% of the specimen immersed in the CSL solution. On the other hand, we noticed that presence of CSL positively influenced the mass losses caused by copper sensitive *G. trabeum* and *P. monticola* to the impregnated specimens (Table 3). This could be due to the high amounts of nutrients on the surfaces of the specimens.

The respiration and baiting results indicate that although the wood decay fungi were able to colonize the surface of the treated specimens when immersed in nutrients, they had difficulties in growing to the center of the specimens. There could be several reasons for these observations. First, after immersion in the nutrient

Table 4
Percentages of colonized impregnated specimens exposed to the fungi for 2, 5, 8 and 12 weeks

| Fungus | Percentages of colonized specimens (%) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 weeks | | | | 5 weeks | | | | 8 weeks | | | | 12 weeks | | | |
| | C | G | CSL | CSL + G | C | G | CSL | CSL + G | C | G | CSL | CSL + G | C | G | CSL | CSL + G |
| Av | 0 | 0 | 0 | 0 | 33 | 66 | 33 | 33 | 66 | 66 | 66 | 33 | 100 | 100 | 100 | 100 |
| Lp | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 0 | 0 | 66 | 100 | 100 | 100 | 100 |
| Pm | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gt | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Prior to the fungal exposure, samples were immersed in water (C), aqueous solution of glucose (G), corn steep liquor (CSL), or glucose and corn steep liquor (CSL + G).

solution, the fungi have food sources available on the surface of the specimens; thus there was less need for them to grow to the central parts of the wood. Second, the distribution of preservative in the impregnated wood is not uniform. It has been demonstrated that the highest distribution of biocidal components is not on the surface of the specimens, but 1–3 mm below the surface (Mazela, 2000). Therefore, the fungi can easily colonize the surface, but cannot penetrate through the biocide barrier. Third, it can be seen from Fig. 2 that immersion of the CCB-treated specimens in the CSL positively influenced colonization by the copper sensitive and negatively influenced colonization by the copper tolerant fungal strains. One of the reasons for the observed differences could be due to the fact that copper tolerant brown rot fungi produced less oxalic acid in the nitrogen-rich environment (Jarosz-Wilkolazka and Gadd, 2003) so that copper remained in the original-fungitoxic form and, therefore, protected the specimen against decay. It has been reported in the literature that copper in the form of copper oxalate is not soluble and therefore not freely available (Green III et al., 1991; Humar et al., 2004; Gharieb et al., 2004). The last possible reason for the reduced colonization could be that in the presence of high concentrations of nitrogen compounds, formation of insoluble copper oxalate was not possible but new soluble and therefore fungitoxic complexes of copper, oxalic acid and nitrogen containing substances similar to those reported for copper in the presence of ethanolamine (Humar et al., 2002) could be formed.

We believe that the potential for bioremediation technologies is enormous, particularly in countries where incineration technologies are less developed or banned, such as Denmark. However, much work still needs to be done prior to scale-up of bioremediation processes. In earlier research efforts, many tolerant fungal strains were identified. In this research, glucose was found to be a suitable carbon source, but the use of glucose is not economically feasible due to its high price. Thus, new carbon sources that will promote fungal growth need to be examined. CSL was not found to be a very efficient nutrient supplement; in some cases it even decreased fungal growth. This result indicates that copper tolerant fungi like other brown rot fungi do not require a nitrogen-rich medium for growth. Therefore, further work on the influence of nitrogen on fungal growth and copper tolerance need to be done before scale-up of the bioremediation process is undertaken.

## 4. Conclusions

Immersion of the wood samples in glucose significantly improved the ability of copper tolerant, as well as copper sensitive wood decay fungi to overgrow the surface of CCB-treated specimens. On the other hand, glucose did not contribute to the capability of the fungi to grow to the central parts of the impregnated wood specimens. However, immersion of the samples in corn steep liquor (CSL) did not have any positive influence on growth of the fungi on the specimens compared to glucose. In the case of the control specimens, negative influence on colonization and decay abilities of the fungi were observed.

The fastest overgrow of the surface and the fastest penetration to the center of the impregnated specimens were observed at copper tolerant fungus *A. vaillantii*. However, immersions of the specimens into the aqueous solution of CSL influenced negatively the growth of copper tolerant and influenced positively the growth of copper sensitive fungal strains. However, none of the copper sensitive fungi reached the bait in the central part of the impregnated samples even after 12 weeks of exposure.

Bioremediation of copper-treated wood waste is a promising method, but there are still some knowledge gaps that need to be filled prior to scale-up of the process, especially regarding the use of nutrient supplements that will promote the growth of the fungi.

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