IMPROVING OF THE IMPREGNABILITY OF REFRACTORY SPRUCE WOOD BY *BACILLUS LICHENIFORMIS* PRETREATMENT

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In this study it was aimed to improve impregnability of spruce (*Picea orientalis* L.) wood with bacteria (*Bacillus licheniformis* A1) pretreatment, using copper/chromium/arsenic Type C (CCA-C) and copper azole Type A (CBA-A). The effects of *Bacillus licheniformis* A1 on weight loss, copper uptake, and compression strength of samples were determined. Weight loss was slightly changed by bacterial degradation in all test groups. The best copper uptake cases were 1466 ppm for CCA-C and 2730 ppm for CBA-A. Improvement on copper uptake with bacteria pretreatment was in a range of 18 to 103% compared to control samples. Compression strength was decreased by bacterial degradation. However strength losses might be acceptable for several construction applications. *Bacillus licheniformis* A1 seems to have a good potential for increasing the permeability of spruce wood.

Keywords: Bacillus licheniformis; Copper; Impregnation; Refractory; Spruce

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INTRODUCTION

As one of the refractory wood species, spruce wood is hard to impregnate with wood preservative solutions (Ulvcrona 2006). The permeability of Norway spruce (*Picea abies* (L.) Karst) wood depends on its physical, chemical and anatomical properties. The permeability tends to be considerably reduced during drying, which is largely due to permanent structural changes that occur in the wood during the drying process, mainly as a result of the aspiration of bordered pits (Ulvcrona 2006). The permeability decline of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) was found to be less than that of Eastern spruce (*Picea orientalis* (L) Link.) (Usta and Hale 2006). In a previous study, Eastern spruce wood showed lower penetration and retention results, which could be related to their refractory characteristics in comparison to Scots pine, Siberian Scots pine, and Siberian larch (Yildiz 2007). To improve the penetrability of refractory wood, several mechanical, chemical, and biological preparatory methods have been developed. These methods were previously reviewed by Morris et al. (1994), Morrell and Morris (2002), and Panek et al. (2005). Some chemical pretreatment methods were examined to overcome the impregnation difficulties of spruce wood (Militz and Homan 1993; Yildiz et al. 2008). Yildiz et al. (2008) indicated that acid pretreatment with pressure was more

effective than non-pressure method for increasing the retention of some water-borne preservatives in spruce wood. The use of biological agents to improve wood permeability known as “bio-incising” has been intensively investigated for many years. Different fungi have different mechanisms on the extractives and the main components of wood. Pre-treatment with fungi creates space and open flow paths for impregnating chemicals into wood. The bio-incising process depends on wood species, fungal species, and fungal colonization period. In general, after being subjected to bio-incising treatment for a period of time, wood permeability will be increased, but strength or hardness will be decreased (Wan et al. 2006). Keeping the pit membranes permanently in an open state after biodegradation by some bacteria or fungi is one of the keys of biological pretreatment for a better permeability of refractory wood. For biological pretreatments, the permeability of wood can be increased by using certain species of bacteria and fungi (Panek et al. 2005).

Pre-treatment with the white rot fungus *Physisporinus vitreus* is a biotechnological approach for improving the permeability of the refractory wood species such as spruce wood. The process atrributed to "bio-incising" is based on the growth of the fungal hyphae through the tracheids and xylem ray parenchyma. It is reported that fungal activity causes the degradation of pit membranes after incubation period of 6 weeks, and this degredation improves the uptake of liquid substances with small side effect of only negligible losses of impact bending strength (Lehringer 2011). Zhang et al. (2006), investigated a bio-incising treatment using fungus for improving phenol-formaldehyde (PF) resin uptake in aspen specimens. Microscopic examination on treated wood samples showed that fungal hyphae had invaded from cell to cell by eroding the pit membrane, and permeability of treated wood sample was improved owing to the bio-incising.

Apart from investigation the fungal effect on wood permeability, structural changes in wood caused by bacteria were comprehensively reviewed by Clausen (1996). Bacterial pectinase plays an important role in increasing wood permeability, which was believed to be a result of hydrolysis of pectic compounds. In addition, it was mentioned that increasing permeability can be either beneficial by aiding pressure treatment of lumber, or harmful by causing over-absorption of organic preservatives during dip treatments (Clausen 1996). On the other hand, the relationship between water storage time and bacterial activity was deeply discussed for satisfactory impregnation of some refractory wood species such as Sitka spruce and sugi (*Cryptomeria japonica* (D.) Don) (Unligil 1972; Efranjsah et al. 1989; Kobayashi et al. 1998; Panek et al. 2005). Unligil (1972) found that 5-9 weeks was sufficient to obtain full penetration of white spruce (*Picea glauca* (Moench) Voss) sapwood by creosote, and reported a 155% increase in creosote retention by this procedure. He stated that bacterial activity both on parenchymatous cells and tracheids appeared to be the main factor in the increase of penetrability. Efranjsah et al. (1989) demonstrated that the longitudinal permeability of spruce wood was improved after 5 month of soaking in water in the presence of *Bacillus subtilis* due to the attack of bacteria on the torus of aspirated pits. It was emphasized by Kobayaski et al. (1998) that biological pretreatments are economical and ecological, and they do not require special equipment or cause pollution.

In Turkey, Eastern spruce (*Picea orientalis* (L) Link.) is one of the most common wood species grown naturally in eastern Black Sea Region and is intensively used in

forest products industry. Spruce wood is mostly used in the furniture industry and building sector. However, it has limited usages in wood preservation industry because of its difficult impregnability. In many cases, longer pressure duration and higher levels of pressure, which increase the cost of impregnation, are necessary to obtain deeper penetration and uniform distribution in the treated spruce wood.

Bacillus licheniformis has been evaluated as a bioremediation agent for removal of copper, chromium, and arsenic from CCA-treated wood (Clausen 2000). Bacterial treatment is non-toxic for people and environmentally friendly. Under unfavorable conditions it maintains its aggressive properties (Panek and Reinprecht 2011). Additionally, pretreatment by *Bacillus licheniformis* for improving the impregnability of spruce may be a cheap and relatively simple method compared to the other various methods such as incising, boring, using of ultrasound, and chemicals. Furthermore, few data exist on the performance of this bacterium for permeability enhancement in refractory wood species.

The purpose of this study was to investigate the improvement in impregnability of refractory spruce sapwood by *Bacillus licheniformis* pretreatment in different exposure durations. Potential negative effect of bacterial degradation on the compression strength parallel to grain of wood was also investigated.

EXPERIMENTAL

Materials

Spruce wood

Spruce (*Picea orientalis* L.) log was obtained from Trabzon, which is located at Black Sea Region in Turkey. The wood was cut in directions parallel to grain and sawn into samples with dimensions of 2 x 2 x 4 cm (tangential x radial x longitudinal). The mean density of the sapwood was 420 kg/m^3. Straight-grained, defect-free sapwood samples were then placed in an oven at 103°C to determine the oven-dry weight. After, all wood samples were sterilized in an autoclave at 120°C for 30 minutes.

Bacterium Bacillus licheniformis

Bacterial isolate was obtained for the initial screening from Karadeniz Technical University, Department of Biology. *Bacillus licheniformis* (A1 isolate) bacteria was grown on nutrient broth liquid medium comprised of 0.25% (w/v) P (3HB). 500 mL of liquid medium with bacteria were added to every jar has a capacity of one liter.

Bacterial Pretreatment

Sterilized wood samples were added to sterile jar which contained bacteria and nutrient broth liquid medium. Two wood samples were put in every jar. The lids of the jars were not closed tightly. All the jars were stored at 39°C and 60% relative humidity for periods ranging from 1 to 3 months. At the middle of the total test period, approximately 150 mL of liquid medium with bacteria was added to jars to complete the beginning level of liquid medium in the jars.

At the end of every test period, wood samples were removed from jars, washed with sterile distilled water and oven-dried at 103°C.

Weight losses (%) of the samples caused by bacterial attack were calculated based on the initial dry weight for each sample. Then samples were conditioned for 2 weeks at 20°C and 65% relative humidity. Samples were divided into 3 groups. For the first group, samples were exposed to bacteria alone. After exposure to the bacterial pretreatment, samples were impregnated with CCA-C and CBA-A, respectively for the second and third groups. The experimental design is shown in Table 1.

Table 1. Replications of Treatment Groups and Bacterial Attack Durations

Variations	Periods of Bacteria Degradation			Total number of samples
	1 month	2 month	3 month	
Bacterial degradation	10	10	10	30
Bacterial attack + impregnation	20	20	20	60
Impregnation	-	-	-	20
Untreated control	-	-	-	20

Impregnation Procedure

The impregnation procedure was applied according to ASTM D 1413 standard test method. The samples were vacuum impregnated with 2% CCA-C and 2% CBA-A solutions at 760 mmHg at 20°C for 40 min. Afterwards, the samples remained immersed in the solutions for 40 min at atmospheric pressure. The treated samples were subsequently conditioned again for 2 weeks at 20°C and 65% relative humidity.

Copper Analyses

Samples with a dimension of 2 x 2 x 4 cm were cut to two 0.5 cm from both sides. These samples were ground in a Wiley mill with a mesh size of 0.5 mm. CCA-C and CBA-A treated samples were analyzed for copper (Cu) retention in ppm by X-ray fluorescence spectroscopy with a Spectro Phoneix Wood Analyzer.

Compression Strength Parallel to Grain

Compression strength was conducted on the remaining part of the initial sample (2 x 2x 3 cm) according to the TS 2595 standard.

Statistical Evaluation

Compression strength results were statistically evaluated by mean values based on One-Way Anova and analyzed by the Duncan test.

RESULTS AND DISCUSSION

Samples showed less than 2% weight loss for 3 different exposure periods (Table 2). Weight loss in the samples by bacterial degradation was in a range of 0.25% to 1.68%, whilst the average weight loss of the samples degraded by bacteria was 1.02 %. It is known that bacteria are unable to penetrate into wood cell walls, in which the cellulose and hemicelluloses are protected by the lignin matrix (Daniel and Nilsson 1998). However, conflicting opinions exist on the importance of the role of bacteria in the overall decay process. Bacteria observed attacking the wood structure are often referred

to as either erosion or tunneling bacteria (Clausen 1996). Similar results on weight losses were obtained with Scots pine samples exposed to bacterial degradation by Holt and Jones (1983). They reported that weight loss was consistent with those degraded slowly under anaerobic conditions. They also suggested that the resistance of coniferous wood species to bacterial degradation could be attributed to the presence of known inhibitory substances such as lignin or resinous compounds. In our study, bacteria colonized the test blocks within the first month, and the highest weight loss was obtained in this period. Small weight loss was observed (below 1%) in the periods of 3 months. This might be related to the aging of the bacterial solution. Prolongation of the bacterial attack period can cause aging of the solution and, metabolic activity of bacterial inoculants may influenced negatively.

Table 2. Weight Loss and Compression Strength Values of the Samples Subjected to Only Bacterial Degradation

Periods of Bacteria Degradation	Weight Loss (%)		Compression Strength (MPa)	
	Mean	Sd*	Mean	Sd
1. month	1.68	0.17	45.0[a**]	1.0
2. month	1.14	0.20	45.6[a]	1.5
3. month	0.25	0.07	45.5[a]	1.0
Untreated control group			46.7[a]	0.5
* Sd: Standard deviation				
** No significant difference by Duncan's homogeneity test, p>0.05				

As shown in Table 2, compression strength (CS) of the samples exposed to bacterial degradation was found to be slightly lower than the control samples. However, there was no significant difference between the groups according to the One-Way Anova test ($p>0.05$). The lowest CS value was obtained on the samples exposed to bacteria attack for 1 month (45 MPa), in which the ratio of decrease in compression strength compared to the control group was 3.6 %, and it can be tolerated for several construction applications. Clausen (1996) suggested that bacteria of *Bacillus* show no side effects on wood strength, whereas they solely alter wood permeability. As expected, there was a negative correlation between the weight loss and compression strength decrease. Panek and Reinprecht (2011) found relatively minor effects on mechanical properties after attack of spruce sapwood with *B. subtilis* in accordance with minimal destruction of the cell wall structure of spruce sapwood tracheids. Similarly Unligil (1972), and Efransjah et al. (1989) reported only a minimal decrease on the mechanical properties of spruce sapwood after its bacterial attack. Diamandis and Koukos (1992) stated that mechanical properties of wood such as toughness were not altered after the bacteria treatment. Clausen (2010) noted that bacteria have little effect on wood structure, except over long periods.

Changes in copper content (ppm) and compression strength (MPa) of the samples exposed to bacterial degradation with CCA-C and CBA-A impregnation are given in Table 3 and Table 4, respectively. Compared to control groups, increases in copper content (%) of samples treated with CCA-C and CBA-A followed by bacterial degradation are given in Figs. 1 and 2, respectively.

Table 3. Copper Contents (ppm) and Compression Strength of the Samples Impregnated with CCA-C Followed by Bacterial Degradation

Periods of Bacteria Degradation	Copper content (ppm) Mean	Sd	Compression strength (MPa) Mean	Sd
1. month	1367	5.6	40.5[b*]	1.2
2. month	1466	1.5	40.9[b]	0.4
3. month	1047	21.6	42.3[b]	1.06
CCA-C treated samples	722	4,7	46.0[a]	2.1
Untreated control group			46.7[a]	0.5
*Different letters (a-b) indicate significant difference by Duncan's homogeneity test, p<0.05				

As was shown in Table 3, the highest copper content was obtained on the samples exposed to bacterial degradation for 1 and 2 months. The ratio of increase in copper content compared to the control group was 89% and 103%, respectively (Fig. 1). Some bacteria species such as *Pseudomonas* sp., *Bacillus polymyxa* and *Clostridium* sp. produce cellulases and pectinases that alter wood permeability by opening up the crystalline structure of cellulose microfibrils (Clausen 2006). Our findings agreed with the following studies. Unligil (1972) demonstrated that ponding was a very effective pretreatment method, causing increase in permeability of spruce wood. He found that uptake of creosote oil was increased by 155% after ponding because of bacterial attack, which degraded pit membranes. Efransjah et al. (1989) also reported an increase in the permeability of spruce sapwood after 5 months ponding with bacteria *Bacillus subtilis*. This increment was because the pits of the wood were destroyed by bacterial attack. In another study, spruce wood samples stored in non-sterile water were exposed to the attack of bacteria *Bacillus subtilis* and their coefficient of permeability was increased by 43.5 % over the 6 weeks of bio-treatment (Panek et al. 2005).

It can be stated that bacteria have maintained their liveliness and enzymes activities especially in the first two months from inoculation. A prolonged period can cause aging of the solution, and metabolic activity or survival of bacterial inoculants is negatively influenced by this period. Many workers have demonstrated that 4 to 6 weeks was sufficient for bacteria to penetrate wood structure. Singh et al. (1998a) suggested that bacterial damage was started as early as 2 weeks after ponding, and that after 4 to 12 weeks of ponding, the destruction of pit membranes was widespread. Singh et al. (1998b) also observed that the penetration of coating into the wood ponded for 2 weeks was deeper as compared to control, and that the deepest and most uniform coating penetration was obtained in the wood ponded for 4 weeks or longer. Hedley and Meder (1992) showed that brown stain resulted from bacterial attack occurred on sawn radiata pine timber between 4 and 8 weeks of storage under water sprinklers. It has been theorized that the accumulation of bacterial biomass or the products of bacterial metabolism such as polysaccharide slimes obstruct the pathways in the ultra-structure of wood (Nijdamm et al. 2001). Additionally, the rapid change in n-hexane uptake between 2 and 3 weeks and the surprising drop immediately after were obtained with $1.5cm^3$ of bacteria inoculated Douglas fir sapwood cubes. It was hypothesized that the bacteria could be responsible for the drop in permeability for 4 to 6 weeks ponding period, while a rapid increase in bacterial activity in the blocks was observed (Archer 1985). On the other hand, the highest compression strength was obtained in the 3-month samples. Compression strength of the samples impregnated with CCA-C were similar to the un-

treated controls. As can be seen in Table 3, there was no significant difference between CCA-C treated samples and controls. The total effect of bacterial degradation and preservative treatment affected the compression strength negatively. This evaluation was also important as statistically ($p<0.05$). However, this strength decrease was evaluated as negligible for many usage areas. Compared to the un-impregnated control group, the maximum compression strength loss was 13% (Table 3). This could be attributed to higher uptake of CCA-C solution. As is well known, CCA-C solution has acidic character, and acidity affects mechanical strength negatively. The metallic oxides, commonly used in waterborne preservative formulations, physically react with the cell wall components by undergoing hydrolytic reduction upon contact with wood sugars. This process, known as fixation, oxidizes the wood cell wall components and may reduce wood strength. The CCA waterborne preservative formulations most commonly used today are sufficiently acidic to cause cell wall hydrolysis, and, as with any chemical reaction. Waterborne-preservative treatments were shown to generally reduce the mechanical properties of wood (Winandy 1996; Yildiz et al. 2010).

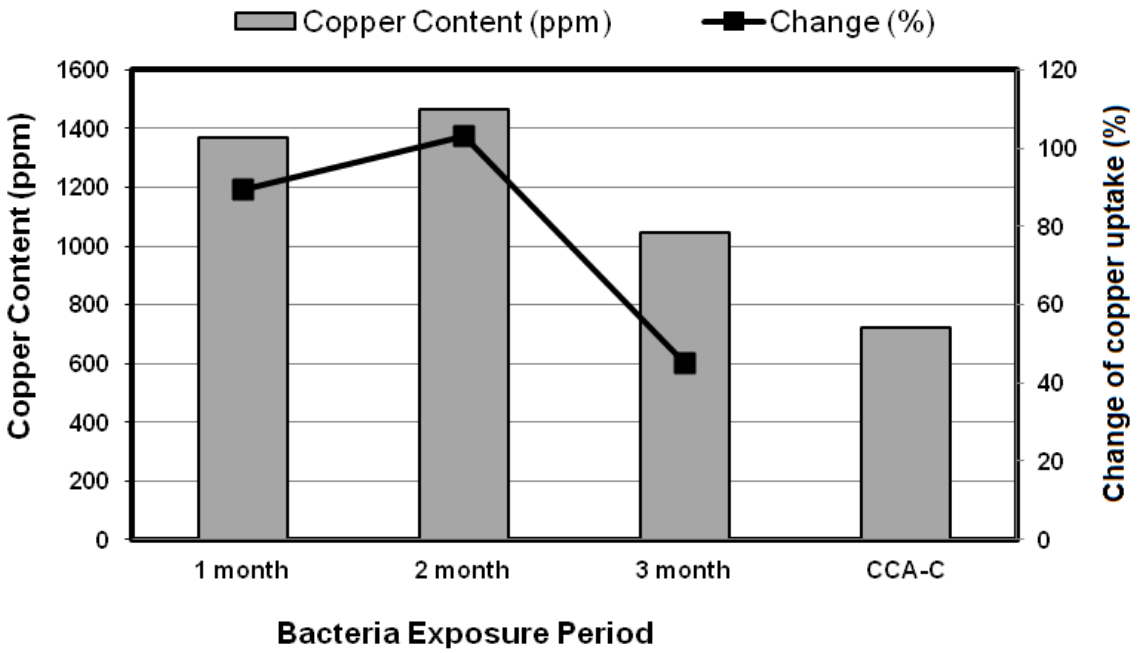

Fig. 1. Copper content of CCA-C treated samples and increase in copper content (%) compared to the controls

Total copper content of CBA-A treated samples was approximately three-times higher than that in CCA treated wood for test samples. However, the effect of bacterial attack on copper content was quite small for CBA-A treatment. For copper content, the best performance was obtained at 1-month exposure (Table 4).

It was observed that the increase of copper content by 18% compared to the control after bacterial attack for one month (Fig 2).

Table 4. Copper Contents (ppm) and Compression Strength of the Samples Impregnated with CBA-A Followed by Bacterial Degradation

Periods of Bacterial Degradation	Copper content (ppm) Mean	Sd	Compression strength (MPa) Mean	Sd
1. month	2730	4.5	44.2[b*]	0.8
2. month	2393	1.9	40.4[d]	0.5
3. month	2257	29.6	42.0[cd]	1.2
CBA-A treated samples	2319	10.8	42.9[bc]	1.2
Untreated control group			46.7[a]	0.5
*Different letters (a-d) indicate significant difference by Duncan's homogeneity test, p<0.05				

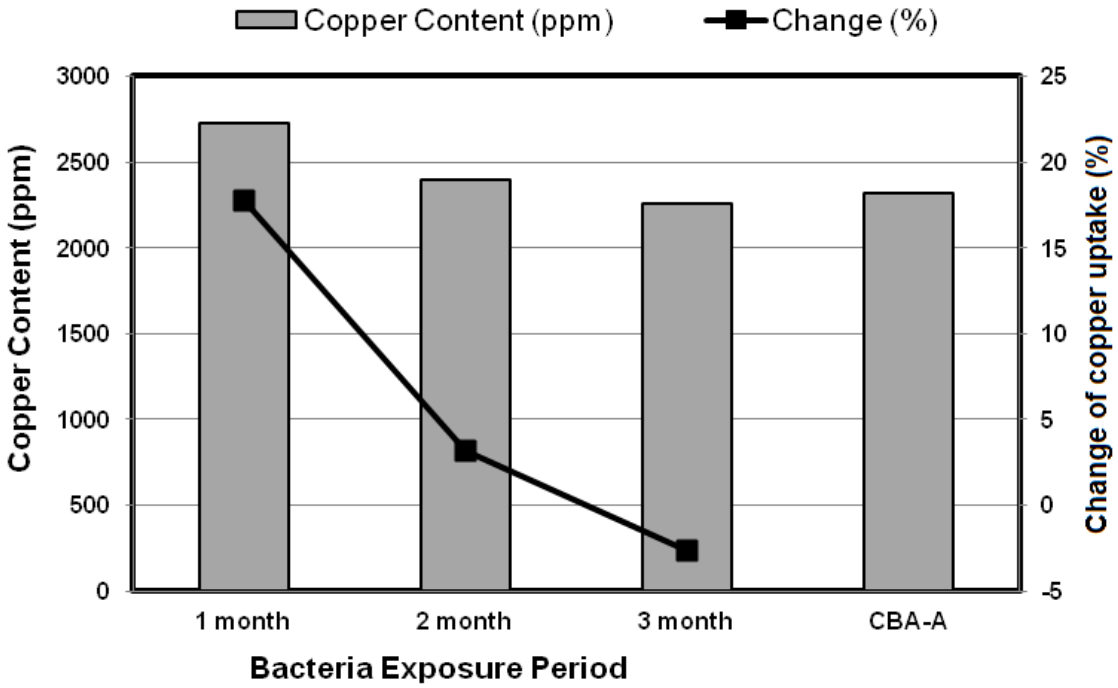

Fig. 2. Copper content of CBA-A treated samples and increase in copper content (%) compared to the controls

The CBA-A formulation has a higher content of copper than CCA-C. In the CCA-C formulation, chromium, copper, and arsenic are 47.5, 18.5, and 34 percent by weight, respectively, whereas copper azole type A (CBA-A) contains 49 percent copper, 49 percent boric acid, and 2 percent tebuconazole (Lebow 2004; Freeman and McIntrye 2008). This is one of the probable reasons for higher total copper content in CBA-A treated wood specimens. On the other hand, copper ethanol amine is used as the copper source in this kind of new generation wood preservatives, which includes ammoniacal copper quat-type D (ACQ-D), copper dimethyl-dithio-carbamate, and copper azole. It was reported that the molar ratio of copper to ethanolamine may influence the penetration, performance of the preservatives and the chemical interaction with wood components (Zhang and Kamdem 1999a). These authors also noted that solutions made from copper hydroxide and copper carbonate yielded higher copper retention (Zhang and

Kamdem 1999b). Consequently, the increase of total copper content in CBA-A treatment is attributed to this chemical structure of the preservative solution.

Retention increases (%) in the samples exposed to bacteria for 1 and 2 month with CBA-A treatment were between 3 and 18 %. Moreover, there was no copper content increase compared to the control after bacterial attack for 3 months (Fig. 2). The chemical structure of the CBA-A solution is believed to play an important role in this scenario. There are two effects of amine in preservative closely related with wood impregnability. First, amine, acting as a chelating agent of copper ion in aqueous solution, plays an important role in copper retention and leaching. Increasing amine to copper molar ratio improves the stability of copper amine complex, and hence increases the transport of copper amine complex into wood. And second, amine to copper molar ratio affects copper absorption greatly in a secondary amine 2-methylamino-amine formulation system.

At low amine to copper molar ratio, copper absorption amplifies rapidly as molar ratio increases, and then copper absorption tends to saturate as the amine to copper molar ratio further increases (Zhang and Kamdem 1999b). CBA Type A used in this study includes 2-aminoethanol carbonate as a secondary amine (URL-1 2011). Accordingly, the slight effect of bacterial attack on copper retention may be due to the saturation of copper absorption in wood with the increase in amine content. On the other hand, there is a little or no information about the retention increase of new generation copper-amine type wood preservatives after bacterial attack. Further studies are needed to understand the relationship between copper-amine type wood preservatives and bacterial attack.

The compression strength (CS) parameters of the samples treated with CBA-A were similar to those found in CCA-C treatment (Table 4). The lowest CS value was obtained at the end of 2-months exposure period (40.4 MPa). One-Way Anova test results indicated that there were some statistically significant differences in the compression strength values ($p<0.05$), showing that treated samples had lower compression strength than the control.

Bacterial and chemical effects on compression strength of the treated samples are summarized in the Table 4 as indicated by letters from a to d. In all groups, compared to the un-impregnated control group, the maximum compression strength loss (13%) was the same as found in CCA-C treatment. As reported before, water-borne preservatives affected the mechanical properties negatively. Greaves (1973) reported that the strength loss of *Eucalyptus regnans* and *Pinus radiata* inoculated with pure cultures of twenty wood-inhabiting bacteria, representing nine different genera, was not considered to be of practical importance. In addition, the softwood species are known to be more resistant to bacterial attack and this phenomenon was attributed to differences in cell wall chemistry, possibly due to the relative lignin contents (Eaton and Hale 1993). Greaves (1971) also reported that some bacteria affect the permeability to liquids of wood, but have no significant effect on strength properties. Clausen (2006) declared that unlike fungi, bacteria cause no structural damage to wood fiber. Ibach (2005) also noticed that bacteria usually have little effect on the properties of wood except over a long time period of exposure.

CONCLUSIONS

1. Weight loss in the samples by bacterial degradation was in a range of 0.25% to 1.68%, whilst the average weight loss of the samples degraded by bacteria was 1.02 %.
2. Pretreatment with bacteria increased the copper uptake of spruce wood by up to 103%, especially in CCA-C impregnation.
3. The effect of bacterial attack on copper content was quite low in CBA-A treatment. The difference on the copper uptake of these two wood preservatives is attributed to the chemical structure of the CBA-A preservative solution.
4. Negligible weight loss in wood samples by bacteria degradation was detected. Slight reduction in the compression strength (CS) of spruce wood occurred as a result of bacterial attack. The loss in CS was judged to be reasonable for several construction applications. Preservative impregnation of bacterial pre-treated samples caused significant decreases on compression strength.
5. The results of this research demonstrate that utilizing of *Bacillus licheniformis* A1 has potential for increasing the permeability of refractory wood species. Further studies are in progress to identify conditions to optimize the rate of permeability enhancement.

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