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Biomodification of *Pinus radiata* Wood to Enhance Penetrability†

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Abstract

Pits form a major pathway for liquid flow in wood. In conifers, such as radiata pine (*Pinus radiata* D.Don), pits between axial tracheids are highly specialised structures, with cell walls overarching a membrane. The membrane consists of a central impervious torus, which is suspended in the pit cavity by a highly porous margo. The bordered pits in radiata pine become aspirated during drying of timber, a condition where the torus becomes lodged against the pit aperture and seals it, thus influencing timber permeability/ penetrability. To enhance timber treatability we have employed an environmentally compatible biological process to remove pit membranes, thus facilitating greater flow of applied wood property enhancing solutions through pits. The biological modification process we employed involved placing freshly sawn radiata pine boards in water in plastic troughs in a glasshouse, and keeping them submerged for periods sufficient for the natural bacterial microflora to colonise wood and destroy pit membranes.

The boards were removed from the troughs after 2-12 weeks and examined by a range of microscopy techniques, including fluorescence confocal microscopy, field emission scanning electron microscopy and transmission electron microscopy, which provided evidence of bacterial colonisation of pit membranes and pit membrane destruction. The microscopic assessment of wood permeability indicated that penetration depth of applied coating was significantly greater in the ponded wood compared with the unponded (control) wood. A fruitful extension of the work presented here would be to develop a biological pre-treatment process using enzymes that will specifically target pit membranes and can be produced cost-effectively for industrial scale operations.

Keywords: biological modification, ponding, *Pinus radiata*, bordered pits, pit membranes, torus, margo, bacteria, varnish, microscopy.

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Introduction

Permeability of wood is of concern for timber processing and utilisation, particularly for species that are difficult to treat with protection agents and property enhancing solutions. Storage of wood in water (ponding) is one of several biological approaches that have been widely employed to enhance permeability of woods, including refractory species and hardwoods, particularly to preservatives. Increase in the permeability of wood from water storage or water sprinkling has been shown to result from bacterial attack on wood. Bacteria colonising wood under wet or water-saturated conditions degrade only the unlignified components, such as pit membranes, thus increasing the permeability of wood (Ellwood & Ecklund, 1959; Liese & Karnop, 1968; DeGroot, 1973; DeGroot & Scheld, 1971; Levy, 1975; DeGroot & Sachs, 1976; Kobayashi et al., 1998), but

ISSN 0048 - 0134

not affecting its strength. This is in contrast to wood-degrading bacteria that can destroy both lignified and unlignified components of wood (Blanchette et al., 1990; Singh & Butcher, 1991; Schmidt & Liese, 1994).

Much is known about the behaviour of pit membranes during water storage of softwoods (Kobayashi et al., 1998; Singh et al., 2000 and references therein) as compared to hardwoods (Liese et al., 1995). In conifer wood, bordered pit membranes consist of cellulose, hemicellulose and pectins, with the torus being rich in pectins and the margo rich in cellulose. Degradation of pit membranes by bacteria under water storage conditions suggests that bacteria produce enzymes needed to degrade and utilise pit membrane components. Support for this view comes from two sources. Firstly, activity assays of enzymes from bacteria isolated from water-stored wood (Fogarty & Ward, 1972; DeGroot & Sachs, 1976) have demonstrated the presence of cellulase, xylanase and pectinase activities. Secondly, studies employing pretreatment of wood with specific individual enzymes or mixtures of enzymes have demonstrated enhanced permeability through destruction of pit membranes (Nicholas & Thomas 1968; Bauch et al., 1970; Tschernitz, 1973; Adolf, 1975; Militz, 1993; Meyer, 1974; Daniel et al., 1996).

Engineering of wood and wood products through biological treatment of wood, such as water storage or enzymatic treatment, holds promise as an environmentally compatible approach for enhancing wood penetrability. This, in turn, may lead to more effective treatment with wood properties enhancing and wood protection agents. The radiata pine wood biomodification work described here is based on the use of a range of microscopic techniques, to enhance visualisation of bacteria and to understand more clearly their spatial relationship to pit membranes in water stored wood. Additionally, increased permeability of ponded radiata pine wood has been demonstrated through application of a coating to the wood surface and subsequent microscopic examination, which showed greater penetration of the coating into ponded wood as compared to unponded (control) wood.

Materials and Methods

Biological Treatment (ponding)

Matched radiata pine (*Pinus radiata* D.Don) boards (300 x 95 x 15 mm longitudinal x tangential x radial) were submerged in tap water in plastic troughs. The troughs were kept in a glass house at around 28 °C. To avoid board surfaces coming into contact, small size wood pieces were used as spacers. The boards were removed 2, 4 and 12 weeks after ponding to examine bacterial colonisation, pit membrane degradation and coating penetration. Two boards were used for each treatment period; one board was kept in a plastic bag for microscopic examination of bacteria and pit membranes, and the other was air dried to study coating penetration after finishing board surfaces with a varnish coating.

Confocal Laser Scanning (CLS) Microscopy

Bacteria in the ponded wood were visualised with a Leica TCSNT CLS microscope using a probe, nitrobenzoxadiazole glycerophosphoethanolamine (NBD-PE), specific for bacteria. Sections obtained using a sliding microtome were treated with NBD-PE and examined according to the methods described by Xiao et al. (2000) for treating sections with the fluorescent tag and imaging with the CLS microscope.

Scanning Electron (SE) Microscopy

Small blocks dissected out from the boards were radially fractured using single edge blades. The fractured pieces were dehydrated in ethanol, air dried and then mounted on stubs. The samples were coated with chromium in a sputter coater and examined with a high resolution SE microscope (JEOL 6700F FE-SEM) at 3 kV to investigate presence of bacteria on pit membranes and pit membrane degradation. To compare penetration of the varnish coating that had been applied to the surfaces of ponded and unponded (control) boards (see the section on coating), thin slices were transversely cut from coated boards using single edge blades, air dried and mounted on stubs. The samples were coated with gold in a sputter-coater and examined with a Cambridge Stereoscan S240 SE microscope at 20 kV.

Pit membrane loss from bacterial attack, and coating penetration were also evaluated by light microscopy. The presence/absence of pit membranes was determined after staining sliding microtome-cut sections with aqueous toluidine blue, which stained lignified cell walls bluish green and pit membranes pink. The depth of coating penetration was assessed in sections that had been transversely cut with a sliding microtome and stained firstly with toluidine blue, then with Sudan IV to enhance the contrast of wood cell walls and the coating respectively. However, micrographs from light microscopy work are not illustrated.

Transmission Electron (TE) Microscopy

Sections cut from ponded wood using single edge razor blades were fixed in 3% glutaraldehyde (prepared in 0.05 M sodium cacodylate buffer) for 3 h at room temperature. The samples were then washed in the buffer, dehydrated in acetone and embedded in Spurr's resin (Spurr, 1969). Sections from unponded wood were similarly processed, except that they were not fixed in glutaraldehyde. Ultra-thin sections from embedded blocks were obtained on a Reichert-Jung Ultracut E ultramicrotome using a diamond knife. The sections were stained in potassium permanganate

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(1% aqueous), according to Donaldson (1992), and examined with a Philips CM 12 TE microscope.

Coating

The varnish coating used in this study was a Nuplex resin primer, Ti Res 1500 as supplied by Nuplex Industries NZ Ltd. The primer was prepared to give a total solids content of 40 % by weight. For a 10 g solution, 5.3 g of Ti Res 1500 was added to a mixture of a hardener (3.0 g Desmoder N 75, supplied by Bayer NZ Ltd) and xylene (1.7 g). The coating was applied by brush at a concentration of about 8 m^2/L. All coated boards were air dried.

Results and Discussion

Bordered Pit Structure and Pit Aspiration

Sectional views of an unaspirated and aspirated bordered pit are shown in Figures 1 and 2, respectively. Figures 3 – 12 illustrate the results of the analytical microscopy undertaken in this study.

In radiata pine sapwood, rays and resin canals are more permeable than axial tracheids, which form the bulk of wood. This is because pits in axial tracheids become completely or incompletely aspirated during conventional drying of timber, which can severely restrict the passage of liquids and liquid borne wood modifying/property enhancing substances across bordered pit pairs. The structure of bordered pits in radiata pine as well as other conifers is highly specialised. The pits consist of a membrane with a

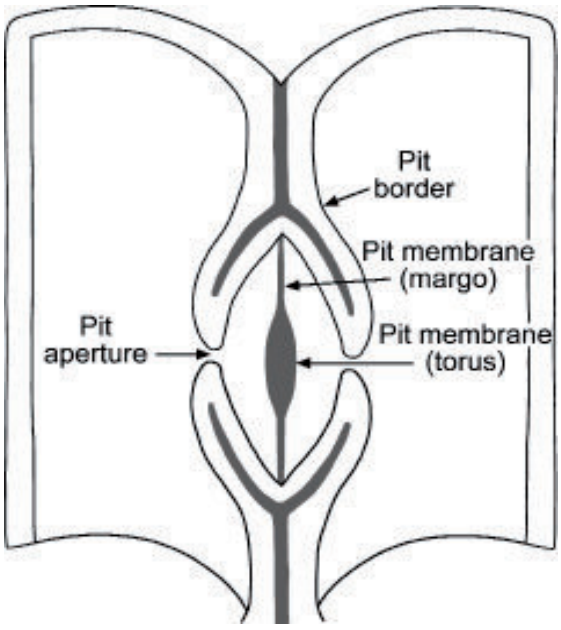

FIGURE 1: Sectional view of an unaspirated bordered pit between adjoining tracheids (diagram).

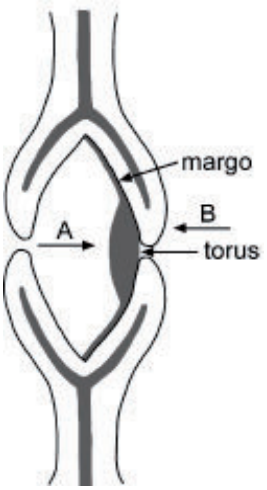

FIGURE 2: Sectional view of an aspirated bordered pit (diagram). SEM views from directions 'A' and 'B' are shown in Figures 4 and 5 respectively.

central torus, which is relatively impermeable, and a highly porous margo that surrounds and suspends the torus within the pit cavity (Figures 1 & 8). Pit aspiration is more common in earlywood than in latewood. In earlywood, the membrane is sufficiently large to deflect under tension from water escaping the wood as vapour during drying, reaching the cell wall of pit borders and coming in close contact with it. This results in closure of pit aperture by the torus (Figures 2 – 5). The membranes of bordered pits in latewood are proportionately smaller and too short to reach pit borders upon deflection during timber drying, thus allowing liquids to readily pass through the porous margo. New Zealand's climate supports fast growth of radiata pine in plantation forests, and earlywood is produced in proportionately greater amounts, making it important to closely examine the relationship of bio-removal of bordered pit membranes

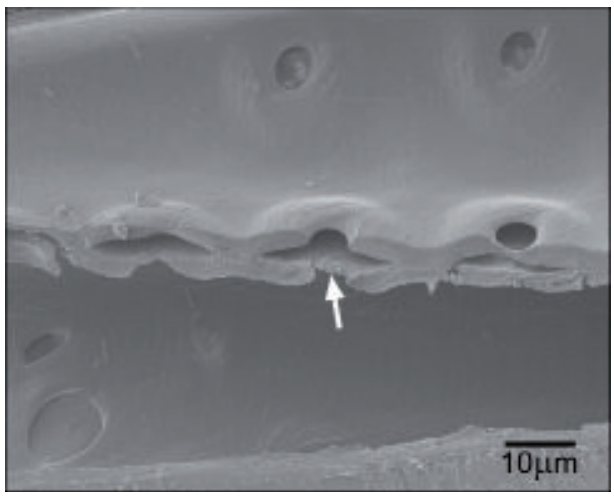

FIGURE 3: Sectional and face views of bordered pits. The arrow points to an aspirated pit membrane. FE- SEM micrograph.

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to enhancement in the penetrability of wood by wood protection and properties enhancing substances. This has been the main focus of the work presented here.

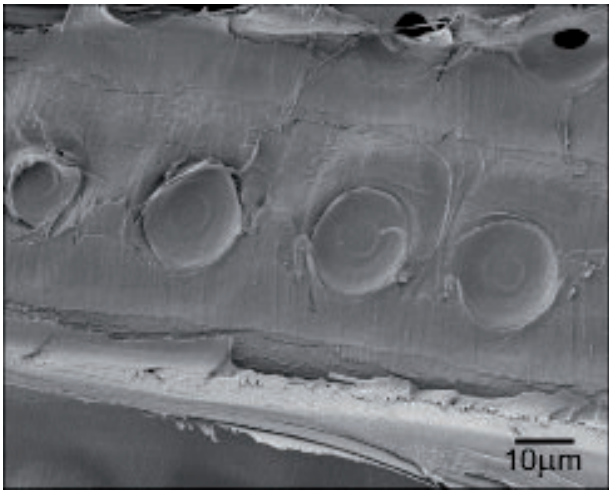

FIGURE 4: Aspirated bordered pits in face view, as visible from the cell lumen side (view 'A', Fig. 2). FE-SEM micrograph.

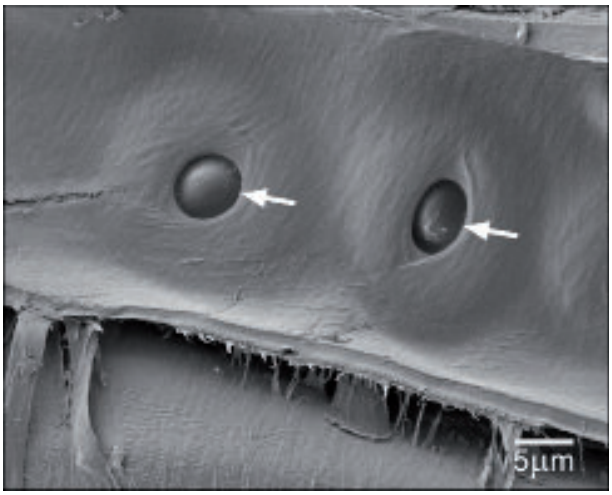

FIGURE 5: Aspirated bordered pits in face view (view 'B', Fig. 2) showing blockage of pit aperture by torus (arrows). FE-SEM micrograph.

Bacterial Colonisation and Pit Membrane Destruction

The biomodification process employed targeted at pit membrane destruction is commonly known as ponding. The radiata pine boards submerged in water were colonised by bacteria within only a few days of ponding. Within two weeks of ponding, bacteria had invaded into subsurface tissue layers, where they were abundantly present on pit membranes (Figure 6). Observations by TE microscopy confirmed their close affinity to pit membranes, as observable in

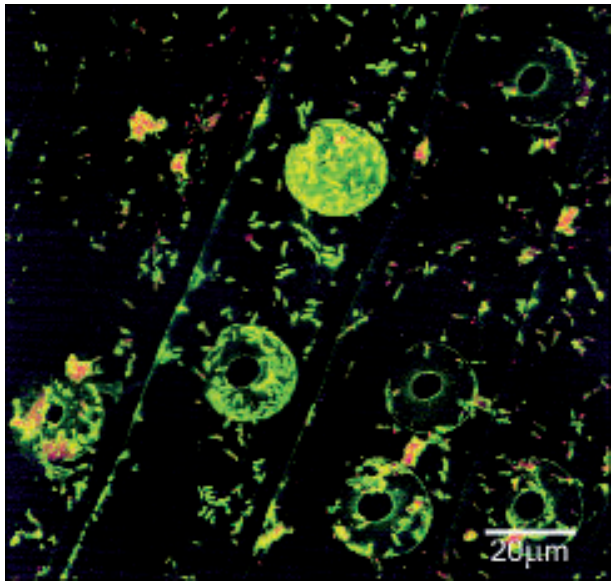

FIGURE 6: Section from ponded wood showing bacteria associated with pits (bright specks). Confocal micrograph.

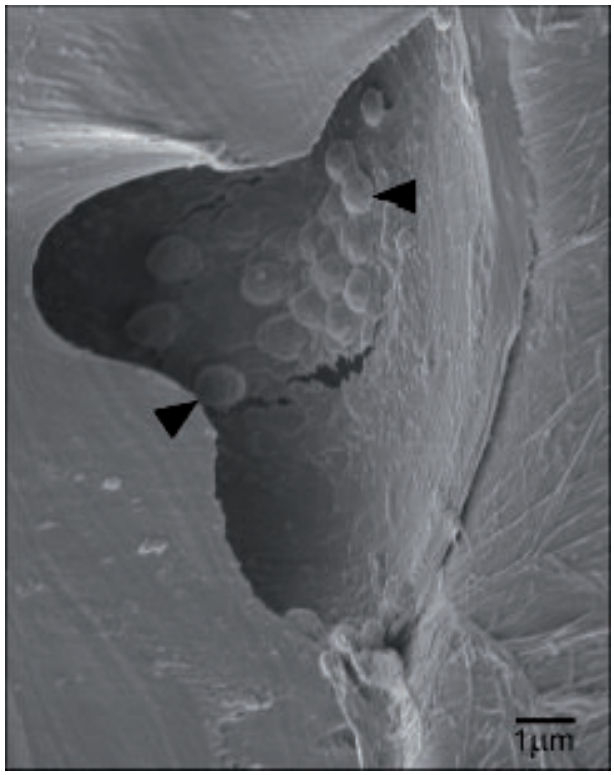

FIGURE 7: Section from ponded wood showing bacteria associated with a pit membrane (arrowheads), which is perforated. FE-SEM micrograph.

Figure 9, where bacteria are mainly present within a pit chamber and are closely confined to the location of pit membrane. Only a few bacteria were present outside of the pit chamber, but they were much smaller than the bacteria located to the pit chamber, and could be scavengers and not the primary pit membrane degraders (Figure 9). The small irregular perforations

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present in the pit membrane in Figure 7 likely represent localised pit membrane degradation. Bacteria were present throughout the membrane surface (Figures 5 and 9) and it is likely that complete pit membrane removal shown in Figure 10 is a consequence of simultaneous attack of the membrane in many places. Furthermore, it is likely that within the mixed populations of bacteria that colonise pit membranes, groups of bacteria are specialised to degrade either pectin-rich or cellulose-rich regions. Both size and morphological differences have been observed, and activity assays of enzymes from bacteria isolated from water-stored wood have indicated presence of a range of enzymes, including pectinase, hemicellulase and cellulase (Fogarty & Ward, 1972; DeGroot & Sachs, 1976). However, direct evidence must await isolation and characterisation of enzymes from specific bacterial isolates as well as immunocytochemical localisation of characterised enzymes.

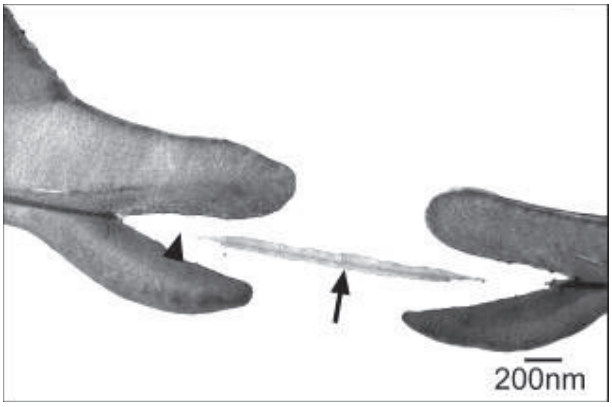

FIGURE 8: Ultrathin section from unponded (control) wood showing a pit pair with a prominent torus (arrow). The margo (arrowhead) is not visible because the stain used, while staining the torus, has not contrasted the margo. TEM micrograph.

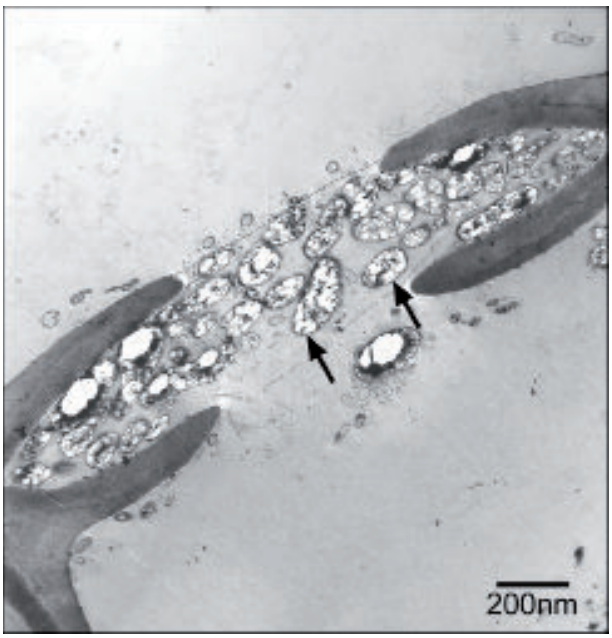

FIGURE 9: Ultrathin section from ponded wood showing a large population of bacteria (arrows) in the pit membrane region. TEM micrograph.

Treatment of wood with commercially available enzymes and their mixtures to degrade pit membranes has also been useful in understanding the structure and chemical composition of bordered pit membranes (Daniel et al., 1996 and references therein). For example, pectinase primarily degraded pectin-rich torus while cellulose-rich margo was degraded by cellulase (Meyer, 1974; Daniel et al., 1996). Partial (Figure 7) and complete (Figure 9) pit membrane degradation was observed in all ponded samples, with degradation taking place in the surface tissue layers in the wood ponded for two weeks and extending to deeper layers in the wood ponded for longer periods.

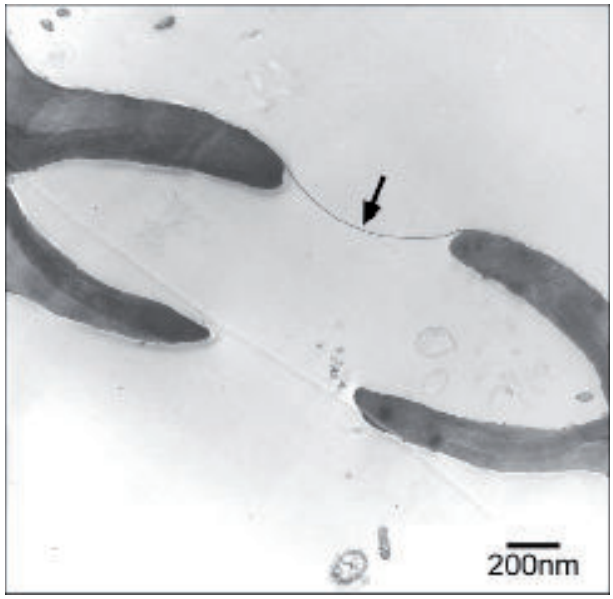

FIGURE 10: Ultrathin section from ponded wood showing absence of pit membrane due to degradation. Bacteria are no longer present in the pit chamber. The material at the arrow could be residue from extractives. TEM micrograph.

Penetrability Enhancement

Bacteria colonising wood during ponding have been reported to degrade all wood components that are not lignified. In addition to pit membranes, cell walls of epithelial cells lining resin canals and ray parenchyma of radiata pine sapwood are not lignified and are also likely to have been degraded during ponding. Therefore, in assessing enhancement of coating penetration due to ponding, contributions from degraded (and hence more highly opened) rays

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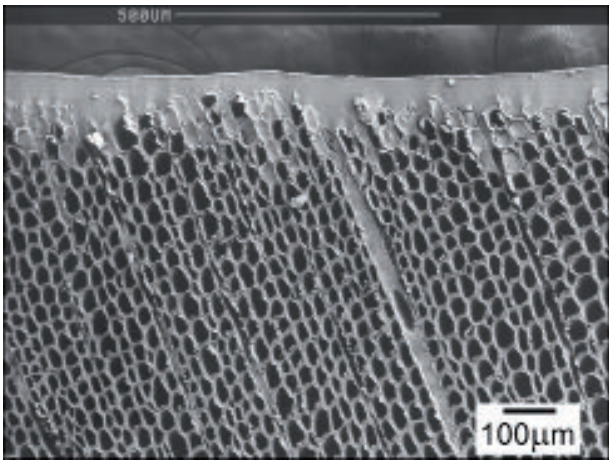

FIGURE 11: Section though varnished unponded (control) wood. Coating has penetrated only a few cells deep. SEM micrograph.

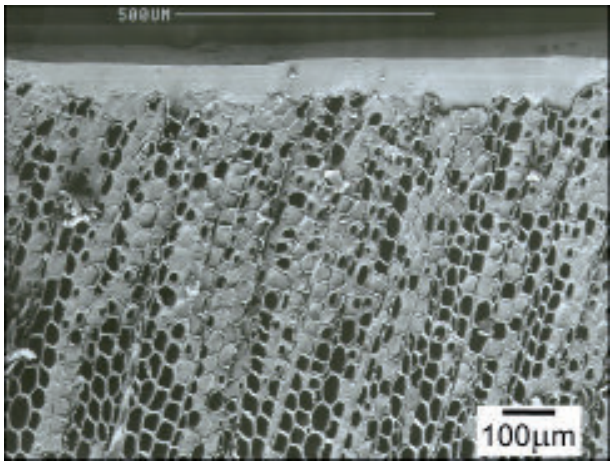

FIGURE 12: Section through varnished ponded wood. Coating penetration is deep. SEM micrograph.

and resin canals must also be considered. However, the focus of our work has been to demonstrate the degradation of bordered pit membranes.

The pattern of coating penetration into ponded wood was consistent with the observations showing pit membrane destruction. The coating penetrated only 2 – 4 cells deep in the unponded wood (Figure 11). In the wood ponded for 2 weeks, coating penetration was up to 8 cells deep (not illustrated), and penetration was considerably deeper in the wood ponded for 12 weeks (Figure 12).

Conclusions

Using a combination of fluorescence and high resolution microscopic techniques, we have demonstrated that during ponding bacteria selectively colonise pit membranes in axial tracheids. These pit membranes are degraded to the extent that they are eventually removed completely. Furthermore, application of a varnish to board surfaces proved to be an ideal system with which to demonstrate penetrability enhancement in radiata pine wood due to ponding. Enhanced penetrability was observed as early as two weeks after ponding, but was much greater after longer ponding periods.

Potential Application

Pits form a major pathway for liquid flow in wood. However, in the earlywood of conifers pits in axial tracheids become aspirated during kiln drying, restricting tracheid-tracheid flow of liquids. Removal of pit membranes enhances permeability, and ponding of wood has been one of several approaches undertaken to achieve this. Although environmentally compatible, ponding cannot however be an option as an industrial-scale process, because of the extended time needed for pit membrane removal and apparent lack of control over consistent reproducibility. The use of enzymes is still an attractive method for enhancing wood permeability to ultimately develop an industrial-scale process however. Over the last decade or so, considerable advances have been made in producing tailor-made enzymes using genetic engineering approaches, and this technology can be employed for producing enzymes that are specific and can be produced at a large enough scale to be cost effective. The use of enzymes produced in this way is likely to prove to be more effective way of pit membrane removal in terms of uniformity of membrane destruction, reproducibility of the process and control over time. It would then be possible to treat wood of all types, including heartwood, with protection and properties enhancing substances (of a wide range of molecular weights and sizes) uniformly and to a desired depth, suitable for treatment of veneers or an envelope treatment of solid wood.

References

Adolf, F. P. (1975). Über eine enzymatische Vorbehandlung von Nadelholz zur Verbesserung der Wegsamkeit. *Holzforschung, 29*, 181-186.

Bauch, J., Liese, W., & Berndt, H. (1970). Biological investigations for the improvement of the permeability of softwoods. *Holzforschung, 24*, 199-205.

Blanchette, R. A., Nilsson, T., Daniel, G. F., & Abad, A. (1990). Biological degradation of wood. In R. M.Rowell, & R. J. Barbour (Eds.). *Archaeological Wood: Properties, Chemistry and Preservation* (pp. 144-174). Washington, DC, USA: American Chemical Society.

Daniel, G., Singh, A. P., & Nilsson, T. (1996). Ultrastructural and immunocytochemical studies on the window and bordered pit membranes of *Pinus sylvestris* L. In L. A.

 ISSN 0048 - 0134

Donaldson, A. P. Singh, B. G. Butterfield, & L. Whitehouse. (Eds.). *Recent Advances in Wood Anatomy*. (pp. 373-383). Rotorua, New Zealand: New Zealand Forest Research Institute Limited.

DeGroot, R. C. (1973). Permeability of sapwood in longleaf pine logs stored under continuous water spray. *Forest Products Journal, 2*3, 43-46.

DeGroot, R. C., & Sachs, I. B. (1976). Permeability, enzyme activity and pit membrane structure of stored southern pine. *Wood Science, 9*, 89-96.

DeGroot, R. C., & Scheld, H. W. (1971). Biodegradability of sapwood from southern pine logs stored under a continuous water spray. *Forest Products Journal, 21*, 553-555.

Donaldson, L. A. (1992). Lignin distribution during latewood formation in *Pinus radiata* D. Don. *IAWA Journal, 13*, 381-387.

Ellwood, E. L., & Ecklund, E. A. (1959). Bacterial attack of pine logs in pond storage. *Forest Products Journal, 9*, 283-292.

Fogarty, W. M., & Ward, O. P. (1972). Enzyme production by bacteria isolated from water-stored Sitka spruce *(Picea sitchensis)*. *Journal of Applied Bacteriology, 35*, 685-689.

Kobayashi, Y., Iida, I., Imamura, Y., & Watanabe, U. (1998). Drying and anatomical characteristics of sugi wood attacked by bacteria during pond storage. *Journal of Wood Science, 44*, 432-437.

Levy, J. F. (1975). Bacteria associated with wood in ground contact. In W. Liese (Ed.). *Biological Transformation of Wood by Microorganisms* (pp. 64-73). Berlin, Springer.

Liese, W., & Karnop, G. (1968). Über den Befall von Nadelholz durch Bakterien. *Holz als Roh-und Wekstoff, 26*, 202-208.

Liese, W., Schmidt, O., & Schmitt, U. (1995). On the behaviour of hardwood pits towards bacteria during water storage. *Holzforschung, 49*, 389-393.

Meyer, R. W. (1974). Effect of enzyme treatment on bordered pit ultrastructure, permeability and toughness of the sapwood of three Western conifers. *Wood Science, 6*, 220-230

Militz, H. (1993). Der Einfluss enzymatischer Behandlungen auf die Tränkbarkeit kleiner Fichtenproben. *Holz als Roh-und Werkstoff, 51*, 135-142.

Nicholas, D. D., & Thomas, R.J. (1968). The influence of enzymes in the structure and permeability of Loblolly pine. *American Wood Preservers Association, 64*, 70-76.

Schmidt, O., & Liese, W. (1994). Occurrence and significance of bacteria in wood. *Holzforschung, 48*, 271-277.

Singh, A. P., & Butcher, J. (1991). Bacterial degradation of wood cell walls: a review of degradation patterns. *Journal of the Institute of Wood Science, 12*, 143-157.

Singh, A. P., Schmitt, U., Kim, Y. S., & Dawson, B. S. W. (2000). The knowledge of wood structure critical to understanding process performance. In Y. S. Kim (Ed.). *New Horizons in Wood Anatomy* (pp. 306-314). Kwangju, South Korea: Chonnam National University Press.

Spurr, A. R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research*, *26*, 31-43.

Tschernitz, J. L. (1973). Enzyme mixture improves creosote treatment of kiln-dried Rocky Mountain Douglas-fir. *Forest Products Journal, 23,* 30-38.

Xiao, Y., Wakeling, R. N., & Singh, A. P. (2000). Use of confocal microscopy in examining fungi and bacteria in wood. *Biofouling*, *15*, 231-239.

 ISSN 0048 - 0134