PEER REVIEWED

Effect of enzymatic pretreatment on the diffusion of sodium hydroxide in wood

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ABSTRACT

The effect of enzyme pretreatment of woods relative to the diffusion of sodium hydroxide was investigated. Enzymes were applied to the sapwood of river birch (Betula nigra), *red maple* (Acer rubrum), *southern red oak* (Quercus falcata), *sweetgum* (Liquidambar styraciflua), *sycamore* (Platanus occidentalis), *and southern pine* (Pinus taeda). *The diffusion coefficients were measured in the tangential and longitudinal directions for each species.*

A 6 x 2 x 2 factorial experiment was used to analyze the effects of species, diffusion direction, and enzyme pretreatment on the mean rate of diffusion in wood. The diffusion of sodium hydroxide in the tangential direction was increased by at least 15% in hardwoods and by approximately 60% in southern pine. Longitudinal diffusion was increased by as much as 110% in the enzyme-treated samples of sycamore sapwood and by 90% in enzyme-treated southern pine. These large differences in diffusion are attributed to the dissolution of pit membranes, which are the main resistance to flow of liquids. SEM pictures revealed the disruption of the pit membranes after enzymatic attack.

Application:

The key application is in assisting pulp and paper scientists to better understand the effect that enzymes have on improving the transport processes in the sapwood of several species.

THE PURPOSE OF THIS RESEARCH was to gain a better understanding of the effects of enzyme pretreatment on transport processes in green wood. Most of the previous research concerning the use of enzymes in pulp and paper applications have dealt with the use of enzymes on pulp, including brownstock, bleached, and secondary fibers *(1–6).*

Processes that improve liquid transport in wood are of great interest to the pulp and paper industry. The size, nature, and number of intercommunicating structures in wood have a major effect on movement of water and other materials through wood *(7).* Enzymatic pretreatments can be used to alter these structures, thus improving the penetration of chemical. Researchers have found that hemicellulase enzymes remove small amounts of xylan or glucomannan and thereby increase the micropore size in the cell wall of unbleached softwood kraft pulp *(8).*

Successful implementation of enzymatic pretreatments may provide substantial increases in the diffusion rates of chemicals in wood; this phenomenon could then be exploited for commercial purposes. For example, pulping requires complete penetration of cooking chemicals into the wood chips. Inefficient impregnation results in a higher amount of shives in the final pulp, a higher lignin content at a given yield, and inferior bleachability *(9).* More uniform pulp, fewer rejects, and shorter cooking times are some of the advantages that can be achieved with enzyme pretreatment.

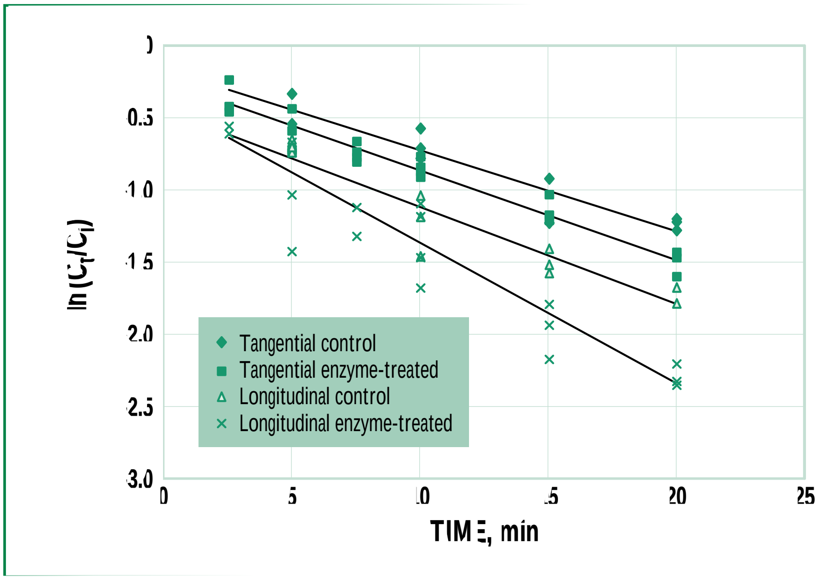
PREVIOUS WORK

Comstock and Côté *(10)* and Meyer *(11)* were able to relate wood permeability to the condition of bordered-pit pairs between longitudinal tracheids in softwoods. Balatinecz *(12)* found that flow in the longitudinal direction in softwoods is from tracheid to tracheid through bordered pits. These pits are concentrated on the radial faces and tapered ends of the fibers. There are 50–300 bordered pits per tracheid *(7).* In pine, transport in the longitudinal direction is primarily through the fiber lumen. The average longitudinal flow is through 3.5 pits/cm, while tangential transport requires transversing an average of 300 fibers/cm. This means that 300 pits in series, which account for all of the resistance to penetration, must be crossed. Pit membranes in most softwood species have a thickened central torus surrounded by a microfibrillar network known as the margo. Because the membrane is flexible, it can shift to one side of the pit cavity, resulting in the blocking of the aperture by the torus.

The dissolution of pit membranes to increase the permeability of wood has previously been investigated. In the timber industry, the ponding process, which involves submerging wood under water for a number of months, is used to increase wood permeability. The bacterial attack of bordered and half-bordered pit membranes serves to make the sapwood more permeable by increasing transport pathways *(13).* Enzyme treatment has also been used to increase wood permeability. Meyer *(14),* Nicholas and Thomas *(15),* and Jutte and Levy *(16)* found that pectinase treatments resulted in dissolution of torus matrix materials, and cellulase addition caused dissolution of margo microfibrils in lodgepole pine. Improved permeability after pectinase treatment was also observed by Nicholas and Thomas *(15),* Bauch *et al. (17),* and Tschernitz *(18).*

The studies previously cited were all investigated using softwoods. It is also of interest to determine how enzyme pretreatments affect transport processes in hardwoods. Vessels that are not occluded by tyloses are generally the most permeable to flow. Tyloses are sac-like membranes that enter the vessels from adjacent parenchyma cells through pit pairs. As in softwoods, pits are the method for communicating between hardwood cells. Pits leading to fibers or tracheids are usually bordered, while those leading to parenchyma cells are usually half-bordered. Similar to softwoods, the pit membrane is continuous across the pit chamber, but tori are extremely rare in hardwood pits. Pit membranes are composed of primary-wall material with a random microfibrillar orientation. Although there are no apparent openings for fluid flow, experimental evidence suggests that hardwood pit pairs are permeable to fluids. Flow may occur between microfibrillar strands *(19)*.

The objective of this work was to evaluate the effect of enzyme pretreatments on pit membrane microstructure and diffusion of sodium hydroxide into hardwoods and softwoods. It was expected that the enzyme preparation would cause degradation of the pit membrane and hence increase transport processes in wood. The existence of a species effect was sought by examining five hardwood species and southern pine.



1. Fractional residual sodium hydroxide (Y_{az}) vs. time for sycamore sapwood

METHODS AND MATERIALS

The species included in this study were river birch *(Betula nigra)*, red maple *(Acer rubrum)*, southern red oak *(Quercus falcata)*, sweetgum *(Liquidambar styraciflua)*, sycamore *(Platanus occidentalis)*, and southern pine *(Pinus taeda)* obtained from an N.C. State experimental forest near Raleigh, NC. Two trees with an average age of 20 years were chosen from each species. Trends in the pulp and paper industry indicate that future wood sources will be composed primarily of juvenile trees. Juvenile trees were used in this study to minimize the extractive content of the wood source.

For these experiments, circular disks were cut with surfaces measuring 47 mm in diameter and 3 mm in thickness. Two sets of disk were prepared; the first set was cut parallel to the axis of the tree, providing a longitudinal surface, while a second set was cut perpendicular to the axis of the tree creating, yielding a tangential surface. Materials entering the tangential surface flowed along the rays in the radial direction. The samples were stored in plastic bags inside a 4°C cold room until used.

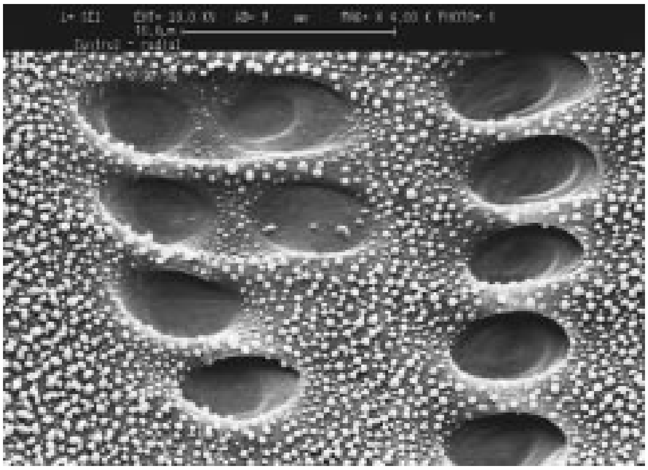
Samples used for scanning electron microscopy (SEM) pictures were split to expose the radial and tangential surfaces and were sputter coated with Au:Pd.

Enzyme pretreatment

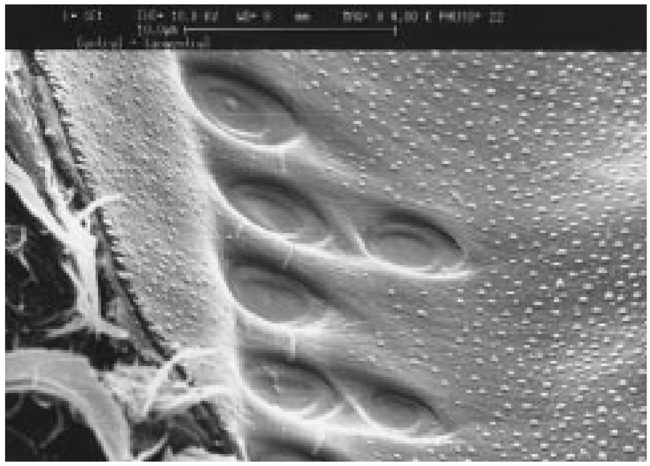
Pergalase A40 (CIBA, Greensboro, NC), which has cellulase and xylanase activity, was applied to samples of each species. Xylanase activity was estimated at 407 U/mL using birch glucuronoxylan as substrate according to the method of Poutanen and Puls *(20)*. Protein concentration was estimated at 175 mg/mL of enzyme solution according to the method of Lowry *et al. (21)*. Cellulase activity was determined using a filter paper assay described by Mandels *(22)*. Cellulase activity was approximately 70 filter paper units (mmol of reduced sugar) per milliliter. The filter paper activity was determined from liberated reducing sugar as measured by the dinitrosalisylic acid method using glucose as a standard.

Pergalase was applied based on the cellulase activity at 5 filter paper units (FPU) per oven-dry (o.d.) gram of wood. The preparation was used with a citrate buffer solution (pH 5) at 50°C. The disks were placed in the enzyme solution for 24 hours to allow time for the solution to penetrate the samples. The enzyme was impregnated into the samples by applying 30 in. of vacuum for 30 min. After enzyme penetration, the disks were washed thoroughly using distilled water.

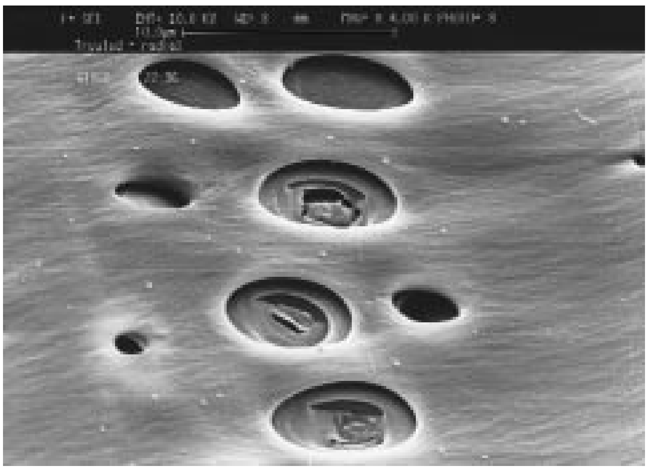
Two control samples were prepared. The first sample was prepared using the procedure previously outlined utilizing a buffer solution without enzyme. A second set of control samples were prepared using only a rinse with distilled water prior to testing. There were no differences in the results achieved with the two samples. Disks used for SEM photos were allowed to air dry in a room controlled at 50% relative humidity. The sides of the disks for diffusion measurements



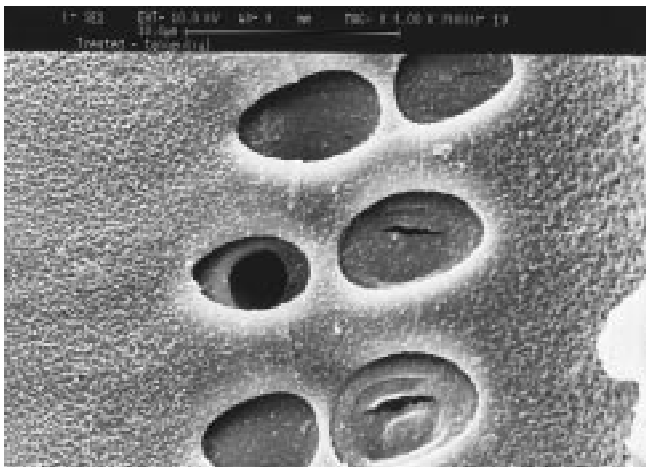
2. Radial view of pits connecting vessels to ray parenchyma cells in a control sample of sycamore sapwood (10 kV x 4)



3. Tangential view of pits connecting vessels to ray parenchyma cells in a control sample of sycamore sapwood (10 kV x 4)



4. Radial view of pits connecting vessels to ray parenchyma cells in an enzyme-pretreated sample of sycamore sapwood (10 kV x 4)



5. Tangential view of pits connecting vessels to ray parenchyma cells in an enzyme-pretreated sample of sycamore sapwood (10 kV x 4)

were coated with liquid plastic and placed in 1.5 N sodium hydroxide. The residual enzyme solution from each sample was tested for sugar content using the 2,4-dinitrosalicylic acid test *(22)*.

Diffusion coefficients

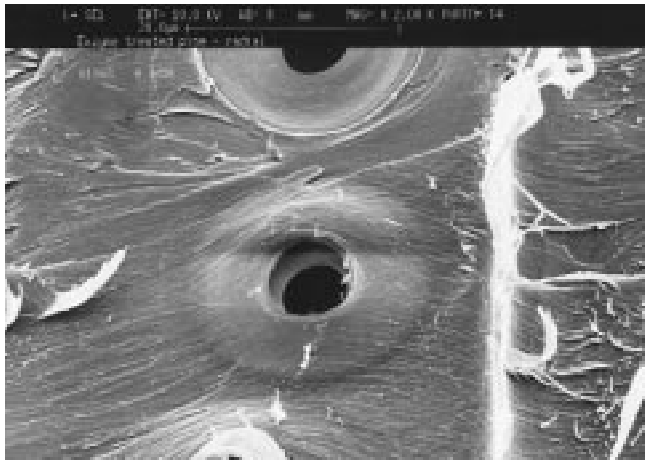
The diffusion coefficient for sodium hydroxide in wood was measured using the technique of McKibbens *(23)* and Talton *(24)*. This involves the use of a form of Fick's Second Law integrated by Newman *(25)* for a block with sealed edges. Newman's equation applies to diffusion through a homogeneous material of constant thickness *L*. The concentration of the solute in the solid has an initial concentration, and the solute concentration at the faces of the block is constant throughout the course of the experiment.

Wood samples were soaked in 1.5 N sodium hydroxide solution for 3 days. Diffusion was restricted to either the longitudinal or tangential direction by coating the sides of the disks with liquid plastic after enzyme treatment. The samples were then removed and suspended in a well-agitated, constant-temperature bath of 0.1 N NaOH. At predetermined intervals, samples were removed and placed in 50 mL of distilled water. The water was titrated using 0.1 N HCl, after allowing the chip to equilibrate for 24 hours. The wood samples were then weighed before and after drying (24 hours at 105°C). This information was used to determine the concentration of NaOH in the wood sample using the following equation:

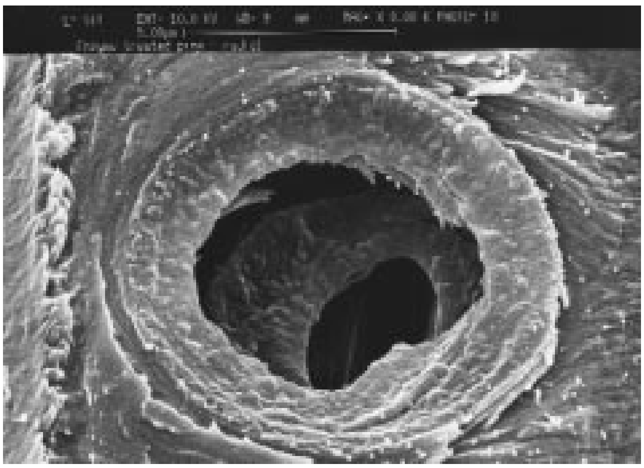
$$\text{Chip NaOH conc.} = C_t = \frac{(\text{mL HCl})\cdot(\text{N Hcl})}{\text{mL titrated sample}} \times \frac{\text{mL } H_2O \text{ in chip} + \text{mL deionized } H_2O}{\text{mL } H_2O \text{ in chip}} \quad (1)$$

The concentration of NaOH inside the chip was used to calculate the fractional residual NaOH concentration (Y_{az}). Y_{az} was calculated as follows:

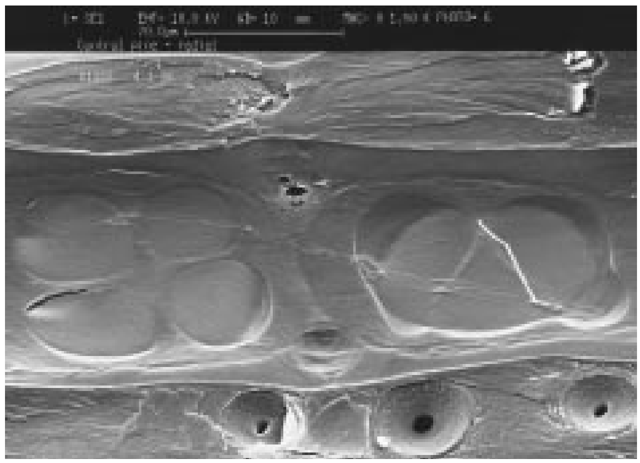
6. Radial view of an intact bordered-pit membrane in a control sample of southern pine (10 kV x 4)



7. Radial view of bordered pit in enzyme-pretreated southern pine (10 kV x 2)



8. Bordered pit in enzyme-pretreated southern pine. The lower pit border is visible because of the absence of the membrane (10 kV x 4)



9. Radial view of crossfield pits in a control sample of southern pine (10 kV x 1.5)

$$Y_{az} = \left(\frac{C_t - C_0}{C_i - C_0}\right) = \frac{8}{\pi^2}\sum_{n=1}^{\infty}\left[\frac{1}{(2n-1)^2}\exp\left(\frac{-(2n-1)^2\pi^2 Dt}{L^2}\right)\right] \qquad (2)$$

where

Y_{az} = fractional residual hydroxide (dimensionless)

C_t = average concentration within the chip at time t, g/cm^3

C_0 = concentration of the solute surrounding the chip, g/cm^3

C_i = initial concentration within the chip at time = 0, g/cm^3

D = diffusion coefficient, cm^2/s

t = time, s

L = thickness of the chip, cm.

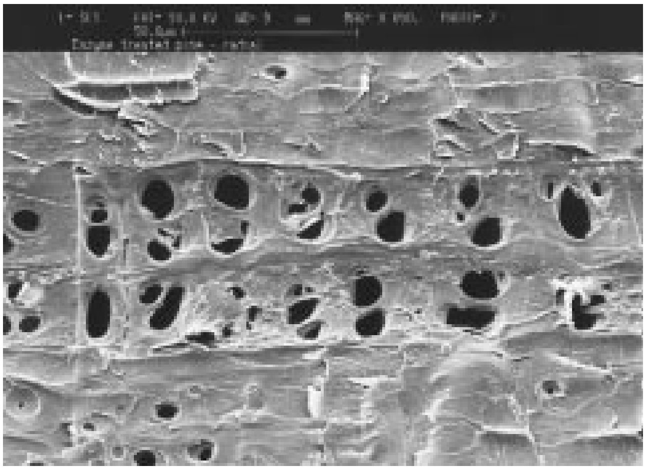
For values of Y_{az} less than 0.6, Eq. 2 reduces to:

$$Y_{az} = \frac{8}{\pi^2}\exp\left(\frac{-\pi^2 Dt}{L^2}\right) \qquad (3)$$

Diffusion coefficients for each sample was determined from the slope of the line formed when plotting the natural logarithm of the fractional residual sodium hydroxide content (Y_{az}) vs. time. **Figure 1** is a graphical representation of the results achieved with sycamore sapwood. The tests were run in triplicate, and each trial is represented by a point on the graph. The diffusion coefficients for the treated and untreated wood disks for all species are summarized in **Table I.**

Results

Microscopic examination. SEM photographs were taken of sycamore and southern pine treated and untreated samples. The two species exempli-



10. Radial view of crossfield pits in an enzyme-pretreated sample of southern pine (10 kV x 650)

fied the dissolution occurring during enzymatic pretreatment with all samples. Five replicates of each sample were observed. The samples were split using a razor to expose the radial and tangential surfaces, which were then sputter coated with Au:Pd.

Figures 2 and 3 are of untreated sycamore sapwood from the radial and tangential directions. Each figure shows intact membranes, bordered pits, and an extraneous material that lined the lumen wall. The extraneous material could be a warty layer that is believed to consist largely of a lignin-like material together with an amorphous carbohydrate *(26, 27)*. Warts may be deposited over normal wall surfaces, helical thickenings, and perforation plate borders and inside pit chambers *(28)*. **Figures 4 and 5** corroborate the hypothesis that enzymes attack pit membranes. These photographs of treated sycamore sapwood reveal that, in enzyme-treated wood, substantial amounts of the pit membranes are missing. In addition, the warty layer observed in the untreated samples was not as distinct, indicating some dissolution of the extraneous material.

A typical pit of southern pine is shown in **Fig. 6.** The membrane is composed of a margo and torus, which cover the pit aperture. After enzyme pretreatment of southern pine sapwood, pits with disrupted membranes are more prevalent (**Fig. 7**). **Figure 8** provides a closer look at the disrupted pits. The lower pit border is visible in this pit, reconfirming that the pit membrane has been removed. Pits connecting ray cells to tracheids are grouped into crossfields. **Figure 9** depicts the pits found in the crossfields of untreated southern pine. Disruption of the membranes of crossfield pits, as shown in **Fig. 10,** was prevalent in the treated samples of southern pine.

Diffusion measurements. Comparisons of the species, pretreatment (i.e., enzyme treatment or no treatment), and direction of application were carried out by analyzing the diffusion coefficients in an analysis of variance (ANOVA) model. It was of primary concern to investigate which of the species had higher diffusion rates and to determine if there was a synergistic effect among the different species, treatment, and direction combinations. For statistically significant effects that were uncovered in these analyses, subsequent pairwise analyses were conducted to better understand the relationships between the factors in this experiment. Adjustments for multiple comparisons were conducted using Tukey's Studentized Range Method.

Analysis of data

Table I summarizes the data that were collected in this experiment. The data were analyzed using the SAS Statistical System *(29)*. Statistical analysis indicates a strong association between the mean diffusion coefficient for both sample treatment and direction. Higher diffusion coefficients for enzyme pretreatment were found in the longitudinal direction. Although sycamore had the highest mean diffusion coefficient, there appears to be no significant differences among the species. Southern pine had the lowest mean diffusion coefficient. These differences were investigated with pairwise comparisons, as previously noted. The species investigated did not show an interaction effect with either sample or direction.

These data were analyzed in a 6 x 2 x 2 factorial design that included all interactions (3 two-way interactions and 1 three-way interaction). It was determined that each of the three factors (species, direction, and sample) is statistically significant at the 99% confidence level (p-values < 0.01). The three-way interaction term was highly nonsignificant (p-value > 0.45) as are the two-way interactions for species by direction and species by sample. Only the interaction of direction by sample treatment is significant.

Pairwise comparisons were made using Tukey's Studentized Range Method to evaluate the mean levels of the diffusion coefficient for each of the species in the above model. At a 95% confidence level, pine had a significantly lower diffusion coefficient than birch, maple, and sycamore. In addition, sycamore had a significantly higher diffusion coefficient than oak. All other pairwise comparisons were not significantly different at the 95% confidence level.

Since the species investigated here do not interact with either direction or sample treatment, the interaction of the latter two factors was investigated. A two-way ANOVA model was fitted

KEYWORDS
Bibliographies, diffusion constant, enzymatic activity, enzymes, microscopy, pits, pores, pretreatment, sodium hydroxide, wood species.

for each of the six species. From the fitted models, the interaction between direction and sample treatment was not significant at the 95% confidence level for all hardwoods. There were no interactions with pine. Separate interaction plots (not shown) for the six species confirmed this. Only pine appeared to have no interactions for the two sets of factor levels.

The significant interaction from the ANOVA analysis confirmed the information suggested by the interaction plots (not shown) for the hardwood species. Both methods indicated that the effectiveness of enzymes is more enhanced in the longitudinal direction. Although there was no interaction found with the southern pine, the interaction plots show that the increase in the mean rate of diffusion was greater in the longitudinal direction. In each case, the enzyme-pretreated samples had higher mean diffusion rates than the controls in the tangential and longitudinal directions.

DISCUSSION

Maple, birch, sweetgum, and sycamore are all diffuse porous hardwoods composed of small-diameter vessels distributed uniformly throughout the annual rings. Oak, however, is a ring-porous hardwood with large-diameter vessels formed each spring. The newly formed vessels are solely responsible for any conduction occurring in the tree *(30)*. Transport processes into the tangential surface of oak are hindered due to the infrequent intervessel pitting found in the solitary vessels *(31)*. Diffusion in the longitudinal direction of the control samples for both the diffuse-porous and ring-porous woods is similar: approximately 7 x 10^{-6} cm^2/s. However, into the tangential surface, the average rate of diffusion in oak is lower (4.71 x 10^{-6} cm^2/s) than the other hardwoods, which have an average rate of diffusion of approximately 6.7 x 10^{-6} cm^2/s. Even after enzyme pretreatment, there was a pronounced difference in the diffusion rate into the tangential surface between oak and the diffuse-porous woods.

Results from this study concur with those found by previous researchers using enzymes on softwoods. Diffusion in southern pine can be increased in softwoods using enzyme pretreatments. Diffusion was increased by at least 40% in each direction. This information is especially important, because southern pine is used extensively in kraft pulping.

The substantial increase in diffusion rate was believed to be a result of disrupting the membranes. This hypothesis was confirmed upon examining the SEM photographs of sycamore and pine. The photographs showed that the enzymes were effective in causing disruption of pit membranes. The substantial increases in diffusion rate suggest that the enzyme pretreatment may affect other structural components in addition to pit membranes. At this point, it is uncertain what other components of the cell wall were affected by the pretreatment. Photographs of both sycamore and southern pine suggest that there may be some dissolution of the surface layer inside the cells. This dissolution could result in lowering the small resistance to flow offered by pit apertures or cavities. Pit cavities and apertures were found to contribute an average of 3% to the resistance to flow in Sitka spruce *(13)*.

The results from this study demonstrated differences in the manner in which some hardwood species react to enzyme pretreatment. Although sycamore had the highest mean diffusion rate, there were no significant differences in the mean diffusion rates for birch, maple, and sweetgum. This indicates that these species reacted similarly in the diffusion study. The significantly lower mean diffusion rate of southern pine, when compared to several of the hardwoods, could be attributed to the low and highly variable diffusion rates found in the tangential direction for southern pine.

Species	Sample	Average diffusion coef., x 10^{-6} cm^2/s	Standard deviation, x 10^{-6}
Birch			
Tangential	Control	6.66	0.345
	Enzyme	7.69	0.241
Longitudinal	Control	7.84	0.236
	Enzyme	13.0	2.25
Maple			
Tangential	Control	6.17	0.666
	Enzyme	7.66	1.18
Longitudinal	Control	7.33	0.170
	Enzyme	14.4	0.707
Oak			
Tangential	Control	4.71	0.648
	Enzyme	6.75	0.829
Longitudinal	Control	7.78	0.773
	Enzyme	12.5	0.863
Sweetgum			
Tangential	Control	6.77	0.535
	Enzyme	7.67	0.938
Longitudinal	Control	7.41	0.302
	Enzyme	12.8	2.07
Sycamore			
Tangential	Control	7.26	0.729
	Enzyme	9.17	0.894
Longitudinal	Control	7.06	0.471
	Enzyme	14.9	0.779
Pine			
Tangential	Control	3.83	0.930
	Enzyme	6.27	2.49
Longitudinal	Control	6.46	0.740
	Enzyme	12.5	1.08

I. Diffusion coefficient of sodium hydroxide in wood samples

CONCLUSIONS

This study on the diffusion of sodium hydroxide in wood pretreated with cellulase/hemicellulase enzyme preparation has shown that diffusion can be increased substantially for some species. Differences in the diffusion between treated and untreated wood samples are largely due to the disruption of the pit membranes. Enzyme pretreatment increased the diffusion rate in the longitudinal and tangential directions of each of the species studied. The extent of the efficacy of the enzyme pretreatment for a particular species was strongly dependent on the direction of diffusion. Increases in diffusion were more pronounced in the longitudinal direction in each species. TJ

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