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Surface Area of Pretreated Lignocellulosics as a Function of the Extent of Enzymatic Hydrolysis

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ABSTRACT

The distribution of surface area as a function of pore size in the pores of hardwood, pretreated by mild acid hydrolysis, was estimated from the pore volume distribution, as a function of the extent of enzymatic hydrolysis. Pore volume distributions were obtained using the solute exclusion technique. Throughout the reaction, the total surface area of the substrate was mostly in pores too small to be accessible to the enzyme, which has been estimated to be 51–90 Å in diameter. For wood pretreated at 200°C, the surface area of pores large enough to be accessible to cellulase decreases rapidly as the enzymatic hydrolysis proceeds. The surface area in pores too small to be accessible to the enzyme decreases more slowly, presumably because the substrate containing these small pores reacts only at the external surface. In wood pretreated at 220°C, a more gradual decrease in the accessible pore surface area occurs. It is hypothesized that this occurs because of the increase of external surface as the size of the substrate particle decreases by more severe pretreatment. This external surface is thought to adsorb more of the enzyme, leaving less for the surface in the pores. For the more severely pretreated hardwood, more enzyme is adsorbed throughout the course of reaction.

Index Entries: Surface area; pore volume; pretreated lignocellulosics; hardwood; enzymatic hydrolysis.

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INTRODUCTION

In order to develop an adsorption based theory for the enzymatic hydrolysis of cellulosic materials, it is necessary to understand the change in surface area as a function of the extent of reaction. The change in surface area during the hydrolysis depends on the structure of cellulose fiber and mode of enzyme attack. If the attack were on an external surface of the fiber, the surface area would decrease as the substrate is consumed. However, if the attack were on the wall of an internal pore, the surface area would increase as the substrate is consumed until the pores began to merge, at which point the surface area would decrease. This behavior of a maximum in surface area has been observed in the case of coal (*1*).

In the case of cellulose, Lee et al. (*2*) have studied the changes in specific surface area of Solka Floc, Avicel, and amorphous cellulose during enzymatic hydrolysis by solvent drying the water-swollen cellulose and using the BET method. They concluded that the specific surface area of the cellulose tested, changes in a manner that depends on the crystallinity of the cellulose. Fan et al. (*3*) have reported that the specific surface area, which was also determined by the BET method, may not significantly affect the hydrolysis rate, and that the decreased rate of hydrolysis in the later stage of the reaction was not owing to a lack of available surface area. However, it is not likely that all surface area estimated from the BET method is available to the enzyme. Indeed, our study of pore volume indicates that most of the area is in pores too small for the enzyme to enter.

The present study was undertaken to measure the relationship between the available surface area, and the extent of hydrolysis of pretreated lignocellulosic biomass. From pore volume distribution it is possible to estimate the available surface area. This surface area may then be compared with the rate of reaction, and the amount of enzyme adsorbed.

MATERIALS AND METHODS

Sources of Substrates, Enzymes, and Chemicals

Mixed hardwood, Wilner 060 (90% birch and 10% maple) was obtained from Wilner Wood Products (Norway ME). Cellulase from *Trichoderma reesei*, GC-123, was obtained from Genencor. All chemicals were of reagent grade and obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise noted.

Substrate Pretreatment

Wilner hardwood was pretreated by acid hydrolysis in a continuous plug flow reactor as described by McParland et al. (*4*). Residence times for a 5% w/w wood slurry containing 1% w/w H_2SO_4 were 8.2 s for a re-

actor temperature of 200°C and 8.7 s for a reactor temperature of 220°C. The pretreated wood slurry was stored in acidified form at 0–5°C.

Enzymatic Hydrolysis

Pretreated wood was prepared for hydrolysis by washing and neutralizing the acidified slurry with distilled water until the pH of the supernatant was between 5–7. In washing the substrate, care was taken to prevent any drying of the material. Hydrolysis was carried out in 158 mL serum bottles containing 95 mL liquid. The initial substrate concentration, S_0, was 5% w/w and the initial concentration of cellulase enzyme was 2.52 mg protein/mL. The reaction was carried out in a shaker bath, at 40°C and buffered to pH 4.8 with 0.05 molar acetate buffer.

Measurement of Hydrolysis Products

Glucose and Total Reducing Sugar concentrations were determined during hydrolysis. Glucose concentrations were evaluated using the hexokinase/glucose-6-phosphate dehydrogenase enzymatic assay (*5*). TRS was determined using the DNS method (*6*). Total sugars as glucose was determined from calibration curves for cellobiose and glucose.

Measurement of Protein in Solution

Protein concentration in solution was determined using the Bradford Protein Assay from Bio-Rad (*7*), using Bovine Serum Albumin as the standard. Absorbances were obtained using the Bausch & Lomb Spectronic 20 spectrophotometer, and concentrations were determined from the standard calibration curve.

Measurement of Pore Volume and Surface Area

Initial pore volume distributions and pore volume distributions of hydrolyzed samples were obtained using the solute exclusion method of Stone and Scallen (*8*). Dextran probes (obtained from Pharmacia) that were 560, 90, and 51 Å were used for the hydrolyzed samples, and probes that were 270, 110, and 8 Å were also used in the initial distribution of wood pretreated at 200°C. Figure 1 illustrates the initial pore size distribution of wood pretreated at 200 and 220°C. After hydrolysis, the reaction mixture was heated to 100°C for 5 min to denature the enzyme. The substrate was then washed with several volumes of distilled water to remove the remaining protein. Four% w/w dextran solutions with 0.5% w/w sodium azide were allowed to equilibrate for 24 h with 0.5–0.9 g dry wt of wet, washed, hydrolyzed substrate. Sugar solutions were separated from the substrate through 0.2 μm nucleopore polycarbonate membrane filters. The solids were washed with several volumes of distilled water to remove any solubles, and were placed in an oven at 65°C to dry. The concentra-

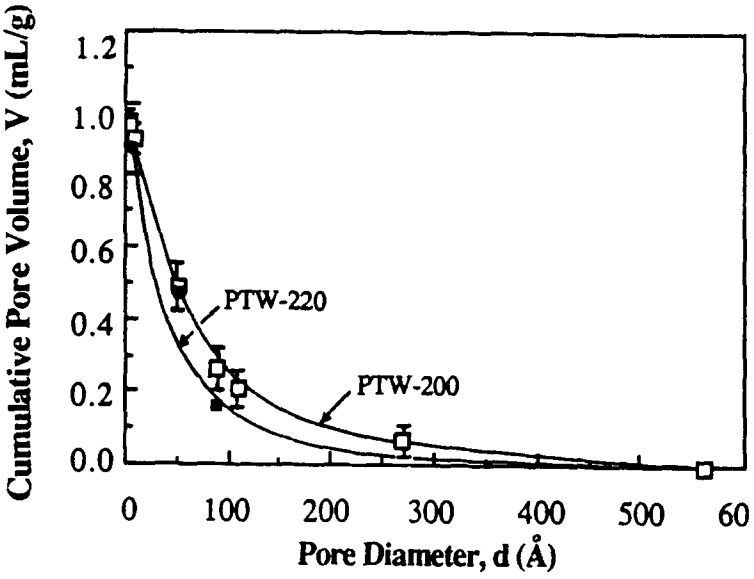

Fig. 1. Cumulative specific pore volume as a function of pore diameter for hardwood pretreated at 200 and 220°C with 1% w/w sulfuric acid for 8.2 and 8.7 s, respectively.

tion of the supernatant sugar solution was determined using a temperature controlled sample cell in a photoelectric spectropolarimeter from Rudolph Instruments. In calculating the specific pore volume, it is the volume that is inaccessible to certain size probes that is determined. An assumption in calculating inaccessible pore volume is that the largest probe, 560 Å, is too large to get into any of the pores. Since the solute exclusion technique measures the volume that is not accessible to probes of certain sizes, it is only possible to calculate the internal surface area of the substrate. The external surface area may not be determined.

Surface area was estimated from the specific pore volume. By assuming a structure of pores, it is possible to calculate the surface area of the wall of pores. In the current analysis, the structure of the pores is assumed to be that of flat parallel plates. In the present experiments we have obtained four points, namely at pore sizes of 4, 51, 90, and 560 Å for the pore volume distribution. Figure 2 illustrates a schematic and typical relationship between the cumulative pore volume and the pore size. A restriction in calculating surface area from pore volume data is that we are differentiating only a few pore volume data points. Ideally, a large number of probe sizes would be used in obtaining a distribution, since this would make the curve more continuous and easier to transform. In an attempt to decrease the error in the estimation of surface area from pore volume data, we assume a straight line between each of the four data points, as shown in Fig. 2. This method allows reproducible calculation of surface area. Estimating surface area from an interpolated curve with a small number of probes, results in a more subjective analysis. In order to

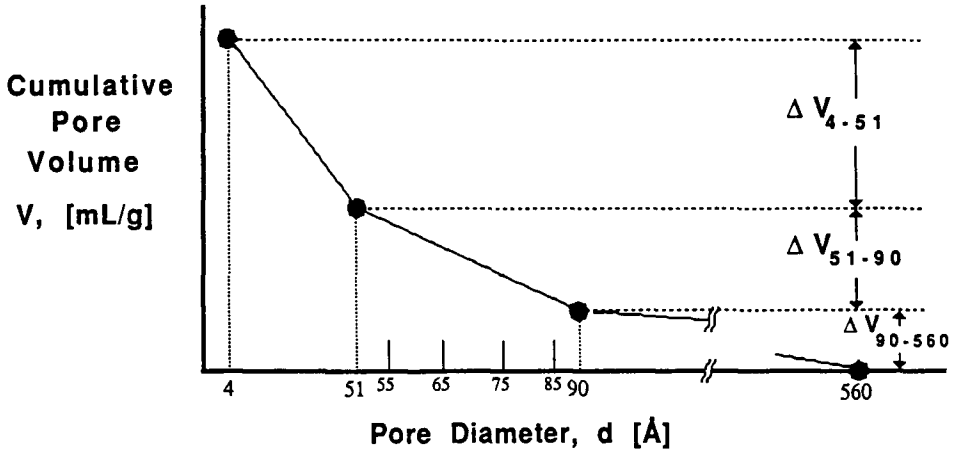

Fig. 2. Schematic diagram illustrating cumulative pore volume vs probe size in calculating surface area. Each pore size region (i.e., 51-90 Å) is divided into equal increments of 10 Å.

estimate the surface area accessible to probes in a certain size range, it is necessary to know the diameter of the pores. If the chosen diameter is too large, the area will be underestimated, and if the diameter is too small, the area will be overestimated. In order to reduce the error, each pore size range is divided into equal increments of 10 Å, where the diameter of each increment is the midpoint of the increment. Eq. (1) illustrates the method used in calculating the specific surface area of pores 51-90 Å in size.

$$\Delta A_{sp}\,[m^2/g] = {}^{(20000)\Delta V_{sp}}\,[mL/g] / 4 * (1/55 + 1/65 + 1/75 + 1/85)\,[1/Å] \quad (1)$$

$$\Delta A_{sp(51\text{-}90)}\,[m^2/g] = 293\ \Delta V_{sp(51\text{-}90)}\,[mL/g]$$

where the average diameter of the pores in this range is 68.2 Å. This calculational procedure is sensitive to the number of increments used when the number of increments is less than 4. Increasing the number of increments from 1 to 3, resulted in average diameters of 70.5, 68.6, and 68.3 Å, respectively. Following a similar analysis for pores in the ranges of 4–51 and 90–560 Å, the average diameter of the pores are 16.6 and 256.4 Å, respectively.

RESULTS AND DISCUSSION

Rate of Reaction During Enzymatic Hydrolysis

The rate of reaction during hydrolysis was determined for hardwood pretreated at 200 and 220°C. The conversion of pretreated wood is presented as a function of time in Fig. 3. The rate of reaction was obtained by calculating the slope at various points on the curves in Fig. 3. The results

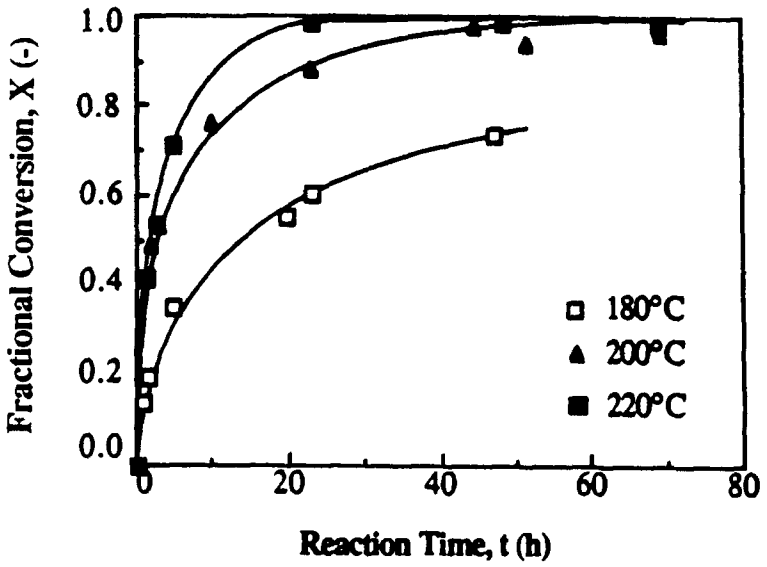

Fig. 3. Fractional conversion vs time of enzymatic hydrolysis for wood pretreated at 180, 200, and 220°C.

of rate as a function of fractional conversion are illustrated in Fig. 4. As is well known (*9,10,11*) the data shows that the degree of pretreatment has an important effect on the rate of hydrolysis.

Changes in Pore Volume and Surface Area During Hydrolysis

Pretreated Hardwood (200°C, 8.2 s, 1% w/w Acid): PTW-200

Pore volume data was obtained on enzymatically hydrolyzed pretreated hardwood, PTW-200. Figure 5 illustrates the results of cumulative specific pore volume vs pore diameter as a function of hydrolysis reaction time. The pore volume accessible to probes larger than 51 Å, decreases rapidly. The data in Fig. 5 may be plotted vs fractional conversion, as shown in Fig. 6. The data points at 0.15 and 0.23 fractional conversions were obtained in a separate hydrolysis experiment in which the initial protein concentration was reduced to 0.252 mg protein/mL (10% the original concentration) in order to slow down the reaction. This data indicates that the pore volume results are reproducible.

The change in specific pore volume may be calculated as follows. The specific pore volume for probes in the range of 51–90 Å, is the difference between the cumulative pore volume for 51 and 90 Å pores. Figure 7 illustrates the results of specific pore volume for pore diameters of 4–51, 51–90, 90–560, and 4–560 Å vs fractional conversion. The pores that are the size of the enzyme, which is assumed to be 51–90 Å (*12*), disappear rapidly, and the volume goes to zero at 50% conversion. The specific pore volume

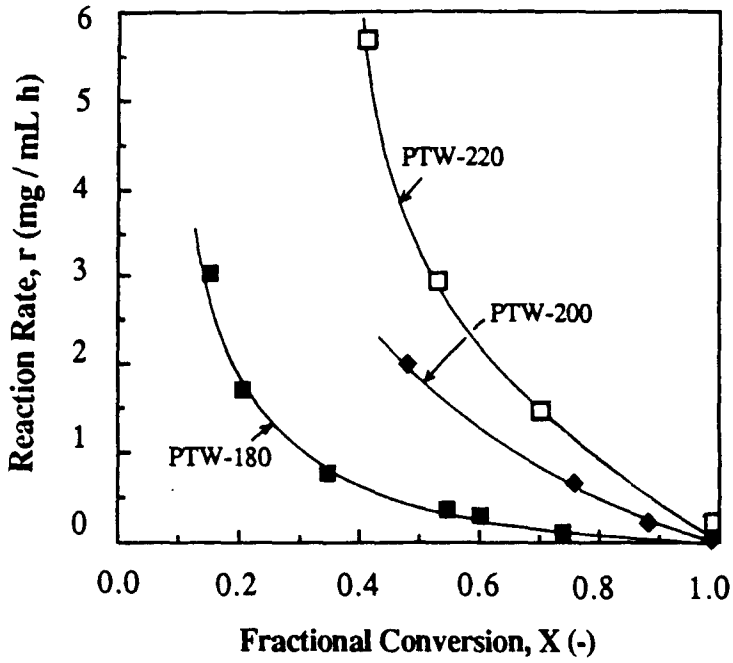

Fig. 4. Reaction rate vs fractional conversion for wood pretreated at 180, 200, and 220°C.

for pores larger than 4 Å experiences a maximum similar to that reported by Lee et al. (*2*) for Solka Floc.

The absolute pore volume is calculated by multiplying the specific pore volume and the residual amount of solid. Figure 8 presents the absolute pore volume for the data shown in Fig. 7. During the initial stages of reaction, the medium pores (51–90 Å) decrease presumably because they are converted into larger pores (90–560 Å). This tends to increase the volume of the larger pores. Since the enzyme cannot enter the pores that are smaller than 51 Å, the smaller pores persist. The pores with a diameter smaller than the size of the enzyme experience a gradual decrease in the absolute volume throughout the course of the reaction, as the substrate is consumed from the external surface.

Following Stone and Scallen (*8*), the absolute surface area was calculated from the pore volume. The results of surface area calculated from pore volume data presented in Fig. 8, are shown in Fig. 9. The data demonstrate an immediate decline in the absolute surface area of pores that are 51–90 Å in diameter. Furthermore, the results indicate that the absolute surface area of the wood is mostly in pores that are inaccessible to the enzyme. These results suggest that the very rapid hyrolysis rate in the initial stages of reaction, correspond to the quick decrease in the larger pores, and that the slow rate of reaction later on during the hydrolysis, correspond to the decrease in the pores that are inaccessible to the enzyme. The rate of reaction is limited by the area that is accessible to the enzyme.

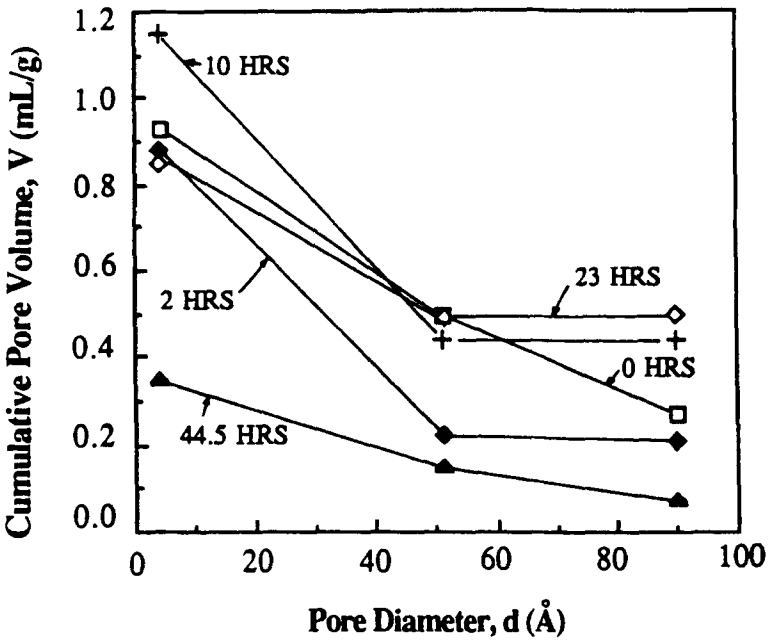

Fig. 5. Cumulative specific pore volume versus pore diameter as a function of reaction time for wood pretreated at 200°C.

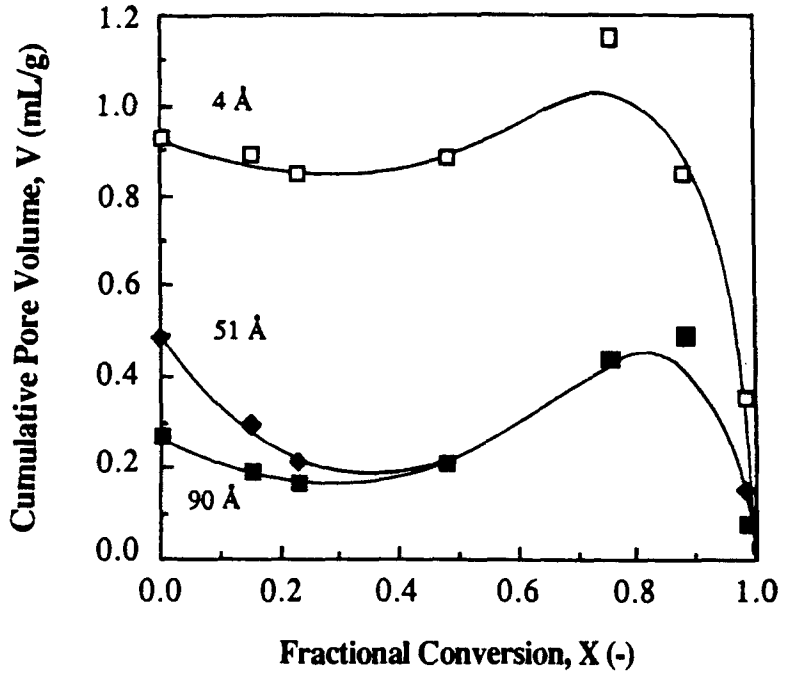

Fig. 6. Cumulative specific pore volume vs fractional conversion as a function of pore diameter for wood pretreated at 200°C.

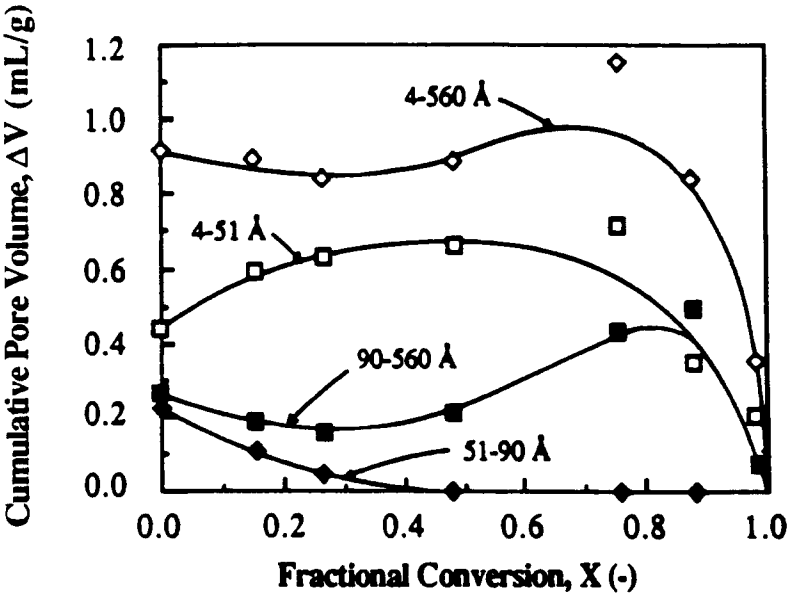

Fig. 7. Change in specific pore volume vs fractional conversion for wood pretreated at 200°C. Specific pore volume is given for the following probe ranges: 4–51, 51–90, 90–560, and 4–560 Å.

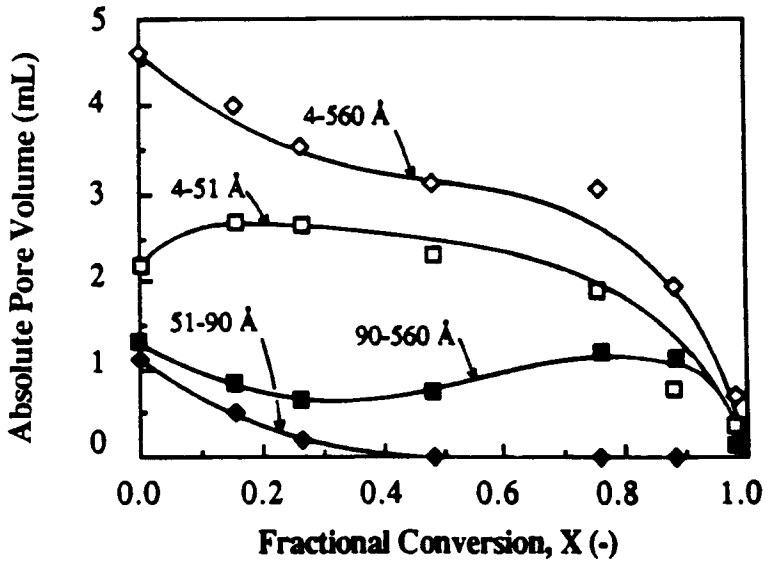

Fig. 8. Absolute pore volume for pores 4–51, 51–90, 90–560, and 4–560 Å, as a function of fractional conversion for wood pretreated at 200°C.

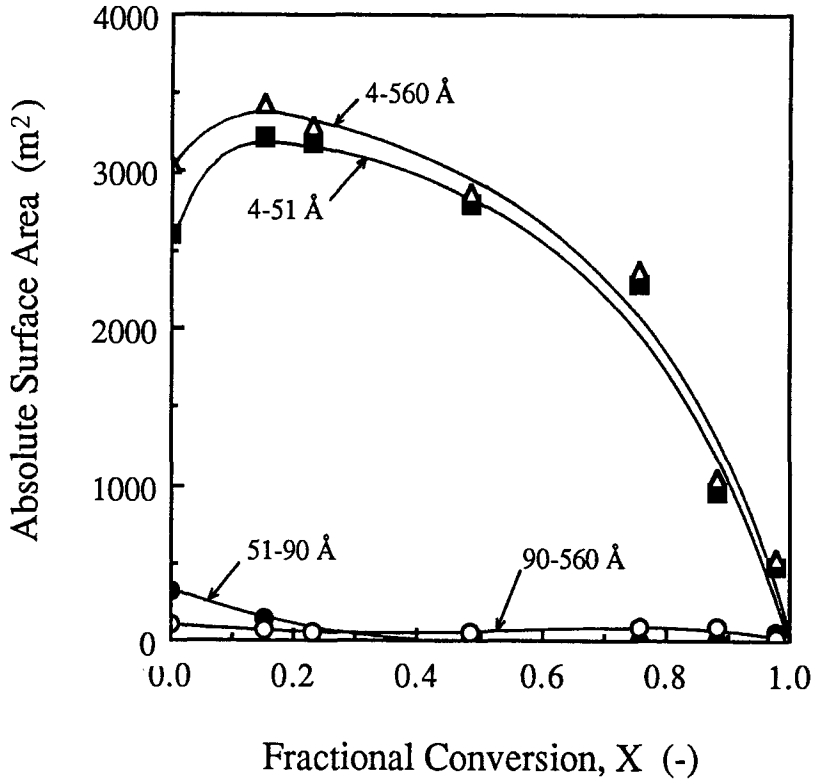

Fig. 9. Absolute surface area for pores 4–51, 51–90, 90–560, and 4–560 Å, as a function of fractional conversion for wood pretreated at 200°C.

The accessible surface in the later stage of the reaction must be the external surface of cellulose fiber.

Pretreated Hardwood

(220°C, 8.7 s, 1% w/w Acid): PTW-220

Results obtained for PTW-220 indicate the same trends as those obtained for PTW-200 only not nearly as exaggerated. Cumulative accessible pore volume vs pore diameter as a function of fractional conversion for PTW-220 is shown in Fig. 10. This corresponds to the data shown in Fig. 6 for PTW-200. The data points at 0.12 and 0.25 fractional conversions were obtained in a separate hydrolysis experiment in which the initial protein concentration was 0.252 mg Protein/mL (10% the original concentration). The volume accessible to 51 Å remains constant during most of the hydrolysis, however, there is an increase in the volume accessible to 90 Å, therefore the volume of pores that are the size of the enzyme decreases. The specific pore volume is illustrated in Fig. 11, which corresponds to Fig. 7. As in the case of PTW-200, there is a gradual decrease in the pore volume of pores with an opening in the range of 51–90 Å. The specific pore volume for pores larger than 4 Å experiences a maximum, as it did for PTW-200, although this maximum is not nearly as defined.

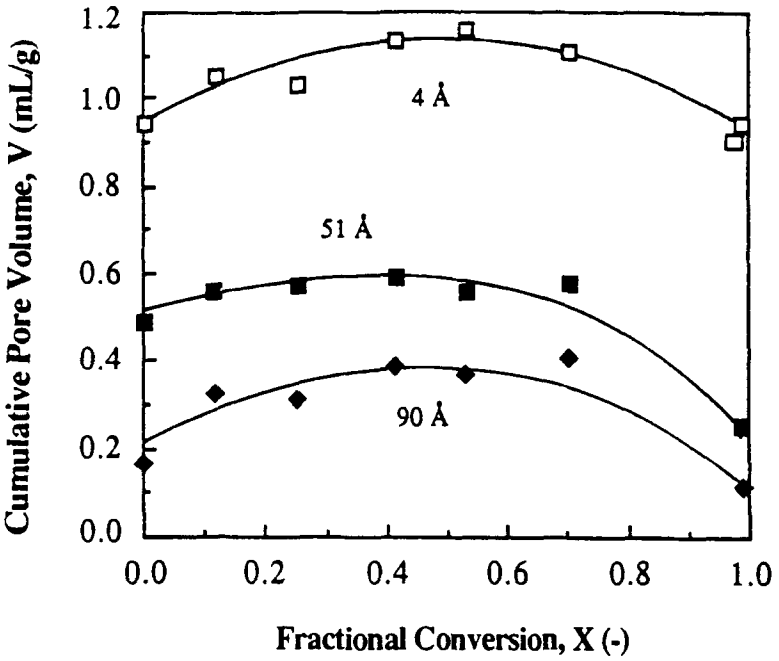

Fig. 10. Cumulative specific pore volume vs fractional conversion as a function of pore diameter for wood pretreated at 220°C.

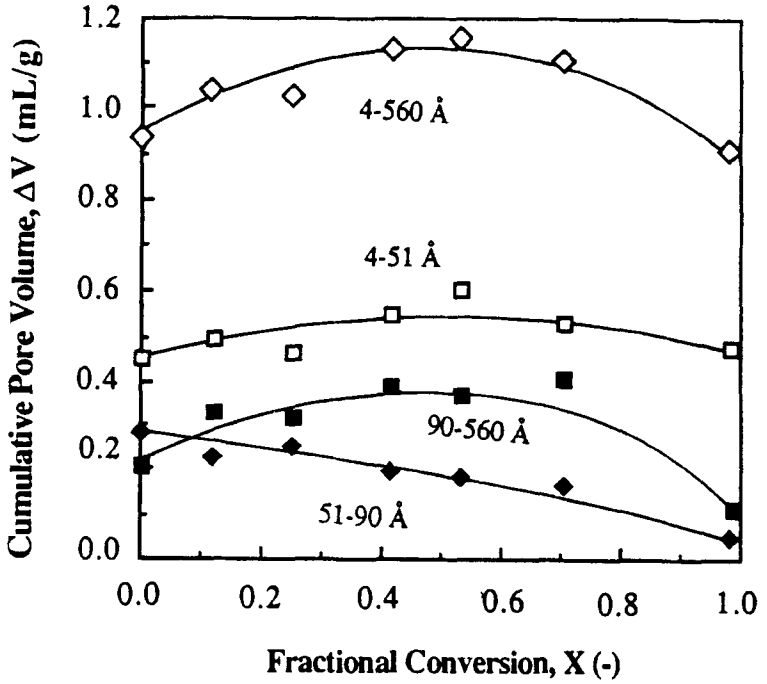

Fig. 11. Change in specific pore volume vs fractional conversion for wood pretreated at 220°C. Specific pore volume is given for the following probe ranges: 4-51, 51-90, 90-560, and 4-560 Å.

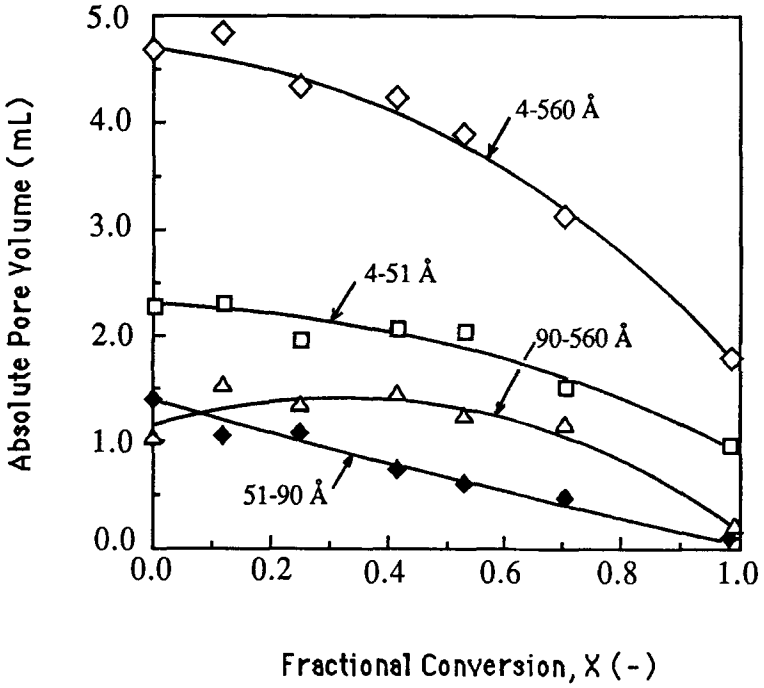

Fig. 12. Absolute pore volume for pores 4–51, 51–90, 90–560, and 4–560 Å, as a function of fractional conversion for wood pretreated at 220°C.

The absolute pore volume was once again calculated based on the residual amount of solid, and is presented in Fig. 12 relative to conversion. Total surface area vs fractional conversion was calculated from this data as previously discussed, and is illustrated in Fig. 13. The results indicate a much more gradual decrease in the accessible surface area than that witnessed for PTW-200. The total area of both substrates is mostly made up of the surface area in the small pores that are inaccessible to the enzyme.

Figure 14 illustrates similar trends in the behavior of the medium sized pores (51–90 Å). The surface area of the medium pores for wood pretreated at 200°C declines more rapidly than the surface area for wood pretreated at 200°C. The slower decrease in the absolute surface area in the medium pores of PTW-220 is possibly owing to the larger external surface of PTW-220. Microscopic observation indicates much smaller particles (data not shown), and hence more external surface area, for the PTW-220 compared to PTW-200. This very accessible external surface area may be attacked more readily than the internal surface area of the accessible pores. The increased external surface area is also consistant with the higher reaction rate for PTW-220 exhibited in Fig. 4.

Figure 15 illustrates differing characteristics in the behavior of the largest pores. Although the surface area belonging to large pores of PTW-220 decreases monotonically, the surface area of the large pores of PTW-200 does not. This difference may be caused by the difference in the rate of decrease of the medium sized pores shown in Fig. 14. In the case of PTW-200, the rate of disappearance of the medium sized pores is so great

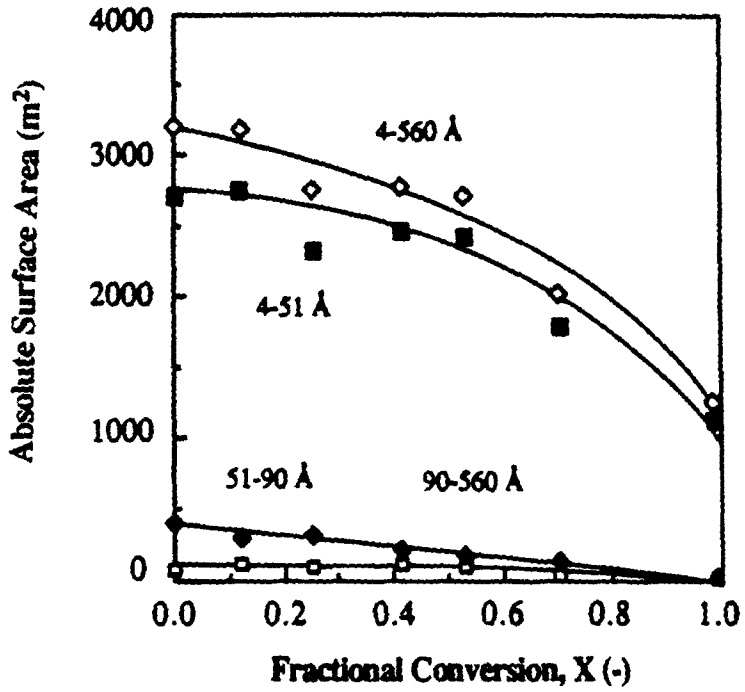

Fig. 13. Absolute surface area for pores 4–51, 51–90, 90–560, and 4–560 Å, as a function of fractional conversion for wood pretreated at 220°C.

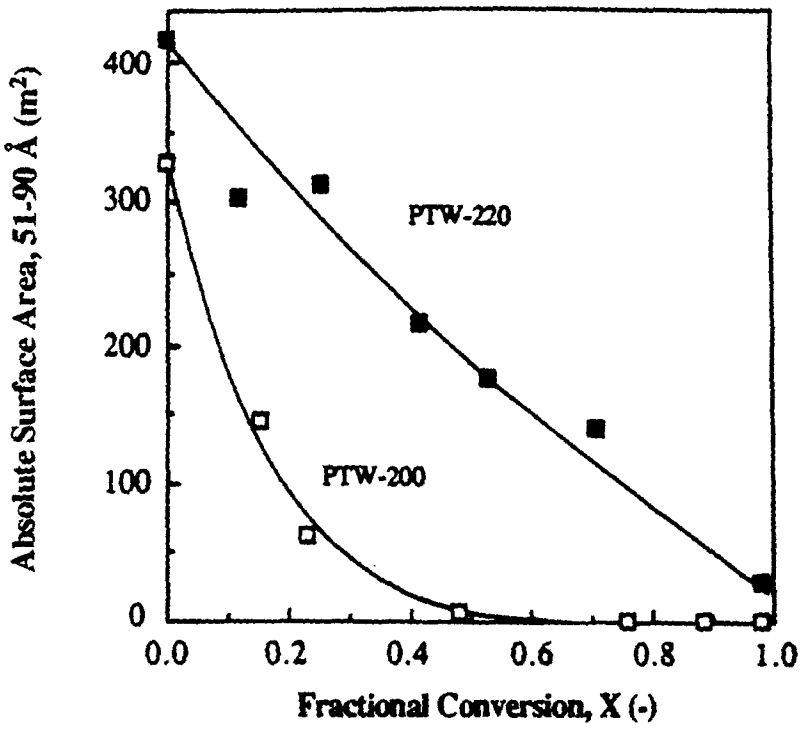

Fig. 14. Absolute surface area for pores 51–90 Å as a function of fractional conversion for wood pretreated at 200 and 220°C.

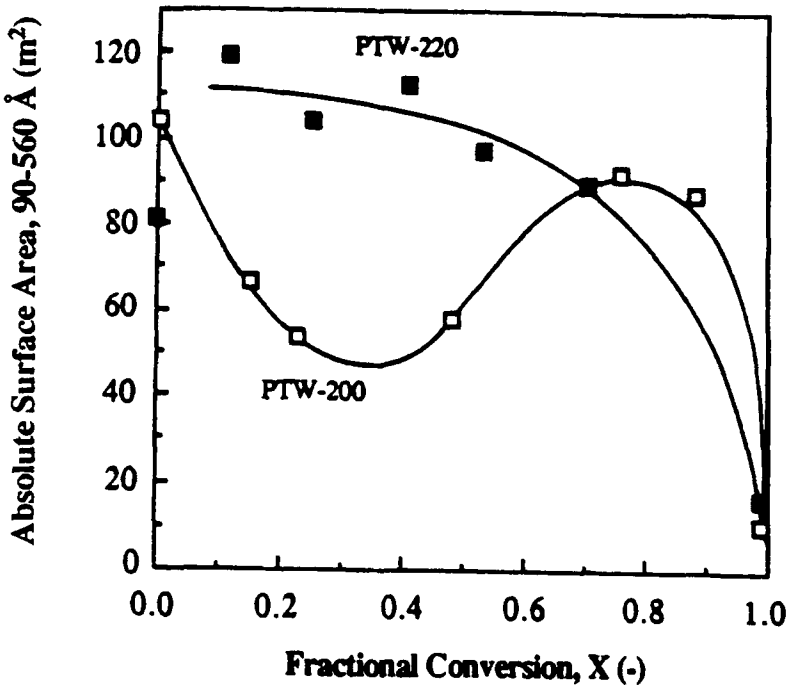

Fig. 15. Absolute surface area for pores 90–560 Å as a function of fractional conversion for wood pretreated at 200 and 220°C.

that the large pores form faster than they disappear, thereby causing an increase in the total surface area of these pores. The medium pores of PTW-220 disappear slowly enough so that the rate of formation of larger pores is less than the rate of disappearance of larger pores.

Enzyme Adsorption During Hydrolysis

The amount of adsorbed enzyme was determined by measuring the total protein in solution and subtracting from the initial concentration. The results for the fractional amount of enzyme in solution as a function of fractional conversion are presented in Fig. 16 for wood pretreated at various temperatures. This data suggest that the higher initial rate of reaction, observed in Fig. 4, for pretreated wood at 220°C is a result of the larger amount of adsorbed enzyme. This also corresponds to a larger surface area being accessible to the enzyme for the more extensively pretreated wood.

CONCLUSIONS

Results indicate that the importance of the accessible pores varies for different degrees of acid pretreatment. For wood pretreated at 200°C, the surface area in the accessible pores decreases rapidly as the enzymatic hydrolysis proceeds. The surface area of pores too small to be accessible

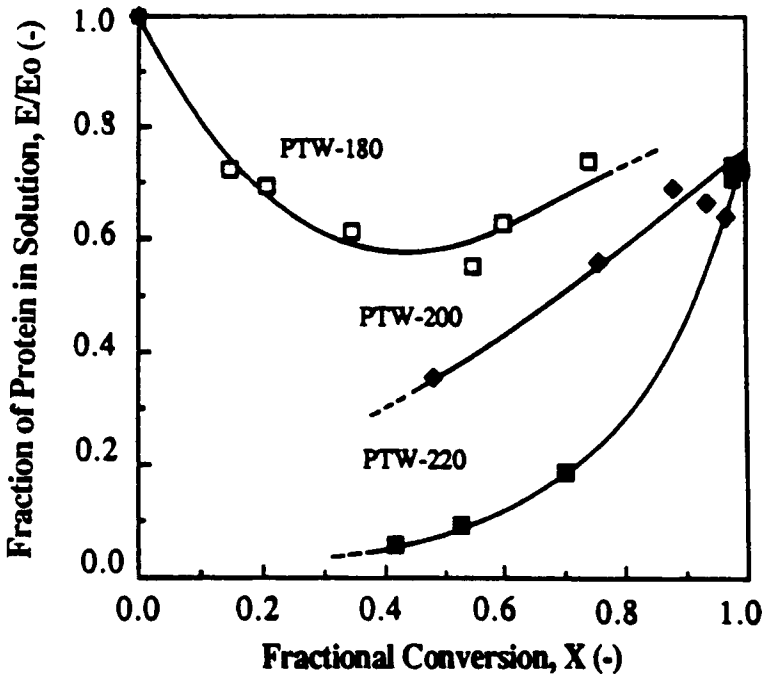

Fig. 16. Fraction of protein remaining in solution as a function of fractional conversion for wood pretreated at 180, 200, and 220°C.

to the enzyme decreases more slowly, presumably because the substrate containing these small pores reacts only at the external surface. In wood pretreated at 200°C, a more gradual decrease in the accessible pore surface area occurs. Severe pretreatment results in smaller particles with more external surface area, and as a result, the accessible internal surface area of the pores is not as critical to the rate of hydrolysis, as it is for less severely pretreated wood. Higher rates were observed for PTW-220 because, with more external surface area, it was not as necessary for the enzyme to diffuse into the pores. For PTW-200, the enzyme must diffuse into the accessible pores, and as a result, the reaction rate is decreased. Also, it has been shown that more available surface area results in greater adsorption of protein. This corresponds to the higher rate of reaction that was observed for wood with a larger available surface area. In order to more accurately characterize the behavior of the largest pores, it is necessary to use probes within the range of 90–560 Å. Results also confirm the importance of the internal surface area. Confirmation of the importance of the external surface area awaits measurement of this surface.

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