Utilization of Waste Cellulose. 7. Kinetic Study of the Enzymatic Hydrolysis of Spruce Wood Pretreated by Sodium Hypochlorite

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ABSTRACT: Spruce wood sawdust has been pretreated with sodium hypochlorite at pH 8 and hydrolyzed into glucose by cellulases of *Trichoderma viride* at different initial concentrations of substrate and enzyme. The initial rate of hydrolysis was shown to obey a Michaelis type equation and the limiting yield is very high. The kinetics are different for the untreated substrate hydrolyzed under the same conditions; low conversion into glucose is obtained even at very long reaction times. The higher reactivity of the pretreated substrate is explained by a significant increase in the number of accessible anhydroglucose units due to oxidation of the lignin network by sodium hypochlorite. Inhibition of the hydrolysis by glucose and by enzyme adsorption is considered. A simple kinetic scheme is proposed to explain the results.

1. Introduction

Lignocellulosic biomass is an important renewable material that can be transformed into chemicals. Its three main components, cellulose, hemicelluloses, and lignin, can be hydrolyzed into hexoses, pentoses, and aromatic compounds. The enzymatic hydrolysis of the cellulose fraction of lignocellulosic materials into glucose has been extensively investigated in recent years.[1] The obtained yield of glucose was shown to be low unless expensive mechanical or chemical pretreatments were used to increase the accessibility of the cellulose microfibrils to enzymes. Lignocellulosic substrates are indeed intricate composite materials made of partly crystalline cellulose microfibrils embedded in hemicelluloses and lignin. This last component has also been suspected to inhibit the hydrolysis of cellulose by adsorption and inactivation of cellulolytic enzymes. The development of inexpensive pretreatments was thus greatly needed.

Recent work in our laboratory[2-4] has shown that the lignin fraction of lignocelluloses is selectively oxidized by HClO–NaClO when the reaction is performed at controlled pH (7–9). Short reaction times (0.5 h) and room temperature are satisfactory oxidation conditions. This pretreatment results in a significant increase in the initial rate and limiting yield of glucose formation. The loss of cellulose and hemicelluloses was low (~10–20%) during this pretreatment. The yield of glucose, which was 0% in the absence of pretreatment, exceeded 50% when pretreatment by HClO–NaClO was used. Hydrolysis by cellulases of *Trichoderma viride* and hydrolysis–fermentation by mixed cultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum* were studied. The pretreatment can be applied to a wide range of substrates, including wood, straw, bark, and bagasse. The loss of cellulose was low with all these substrates. *Eucalyptus saligna*, a very rapidly grown tree, was shown to be a particularly interesting substrate.[4]

The present work is concerned with a kinetic study of the enzymatic hydrolysis of spruce wood. Untreated and pretreated substrate were compared with different initial concentrations of enzyme and substrate in order to get a better understanding of the efficiency of the HClO–NaClO pretreatment and to optimize the initial rate and limiting yield previously reported for the pretreated substrate.[2-4] A simple kinetic treatment of the results is proposed, and the different possible mechanisms of inhibition are discussed.

2. Experimental Section

Spruce wood sawdust as a 3% aqueous suspension at pH 8 was treated with 0.5 M NaClO at 30 °C as previously described.[3,4] The weight loss and the loss in cellulose were 27% and 20%, respectively.

Enzymatic hydrolyses were performed at 45 °C with cellulases from *T. viride* (Onozuka R-10 from Kinki Yakult MGF Co. Ltd.) that had been soaked in 0.1 M citrate buffer for 24 h at room temperature. Standard conditions were 250 mg of substrate and 10 mg of enzyme in 10 mL of buffer. Other concentrations of enzyme and substrate were used, but a volume of 10 mL was used in all experiments. The specific activity of the enzyme was 0.07 IFPU/mg. The quantity of glucose formed was determined by the glucosoxidase method. The relative error in glucose determination decreased from 20 to 10% when the glucose concentration increased from 1 to 10 or more g L^{-1}. Pentoses, hexoses, and cellobiose were analyzed by HPLC on a 25 cm × 0.46-cm i.d. column filled with anion-exchange resin Aminex A-28 in the borate form. A boric acid–potassium borate solution, pH 8.8, was used as the mobile phase. Spectrophotometric detection was done at 570 nm using the sugar complexes with copper 2,2'-bicinchoninate prepared in situ. Further details on the experimental methods can be found in ref 2–6.

3. Results and Discussion

3.1. Formation of Glucose as a Function of Time for Different Initial Concentrations of Enzyme and Substrate. Figures 1 and 2 give the quantity of glucose formed as a function of time from the pretreated and untreated substrates for different initial amounts of substrate (from 2.5 to 50 g L^{-1}) and the same enzyme concentration (1 g L^{-1}). The yield of glucose is given at the right ends of the curves; it was calculated using as 100% the content of potential glucose in the substrate determined by quantitative saccharification (respectively 0.462 and 0.423 g of cellulose/g of substrate for the pretreated and untreated substrates). Figures 1 and 2 show that the quantity of glucose formed as a function of time increases rapidly for short times of hydrolysis and then approaches a limit. The initial rate of glucose formation is higher for greater initial amounts of substrate. The limiting yield is attained more rapidly for low initial amounts of substrate. It is higher (≃100%) for the pretreated substrate, and extrapolation indicates that it is independent of the initial substrate concentration. It does not exceed 15% for the untreated substrate. The quantity of glucose obtained for short reaction times is given in Table I.

Figures 3 and 4 give the quantity of glucose formed as a function of time for the pretreated and untreated substrates at different enzyme concentrations (from 0.5 to 10

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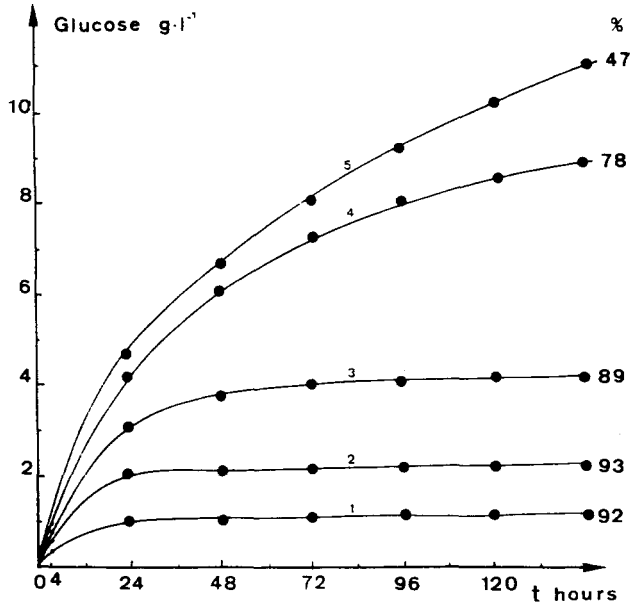

Figure 1. Enzymatic hydrolysis of pretreated spruce wood. Glucose formation as a function of time for different initial wood concentrations in g L^{-1}: (1) 2.5; (2) 5; (3) 10; (4) 25; (5) 50 e_0 = 1 g L^{-1}. Percent conversion of potential glucose is given on the right.

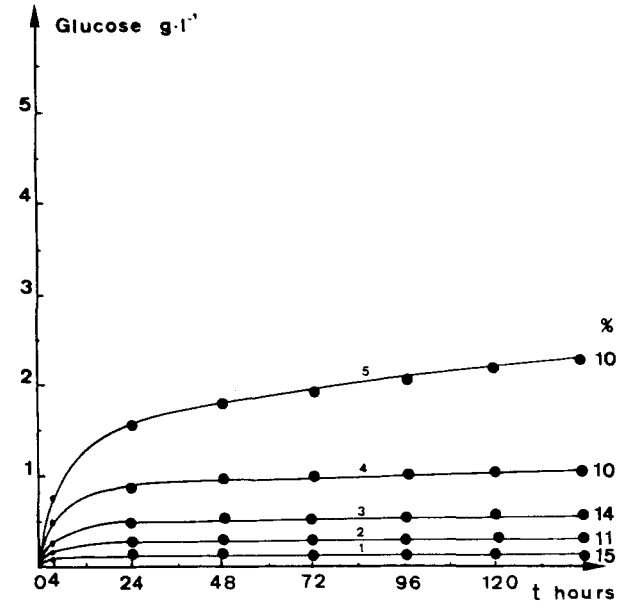

Figure 2. Enzymatic hydrolysis of untreated spruce wood. Glucose formation as a function of time for different substrate concentrations (same concentrations and symbols as in Figure 1).

g L^{-1}). The yield of glucose is given and calculated as in Figures 1 and 2. The limiting yield from the pretreated substrate is almost quantitative (98%) at high enzyme concentrations, and extrapolation leads to a single value at low enzyme concentrations for the pretreated substrate. In the case of untreated substrate, the limiting yield increases with enzyme concentration. For short reaction times (Table I) the quantity of glucose formed increases linearly with time for both types of substrate.

3.2. Formation of Glucose, Cellobiose, and Monosaccharides Derived from Hemicelluloses. The results given in Table II show that the concentration of cellobiose is constant and low when compared to the final glucose concentration after short reaction times (1 h for the untreated substrate and 12–24 h for the pretreated substrate). The quasi-steady-state approximation can thus be used

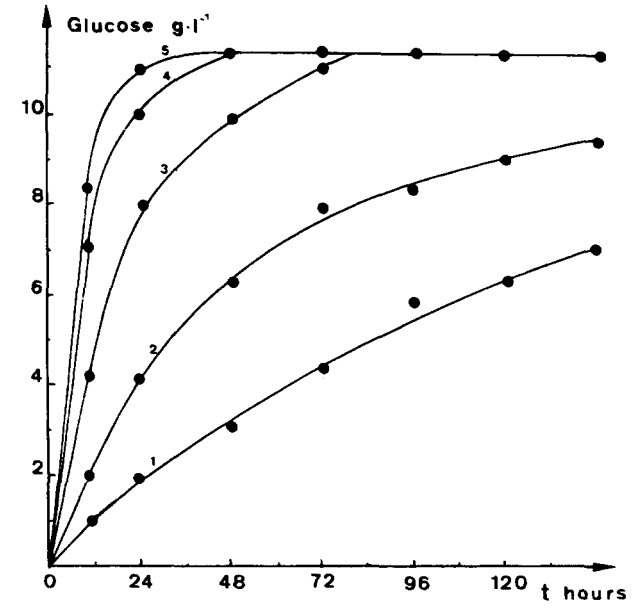

Figure 3. Enzymatic hydrolysis of pretreated spruce wood. Glucose formation as a function of time for different initial enzyme concentrations in g L^{-1}: (1) 0.5; (2) 1; (3) 2.5; (4) 5; (5) 10. Initial wood concentration was 25 g L^{-1}.

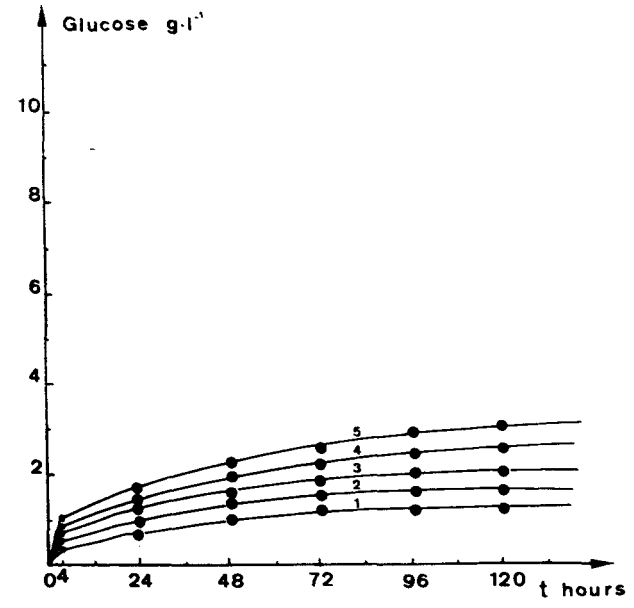

Figure 4. Enzymatic hydrolysis of untreated spruce wood. Glucose formation as a function of time for different initial enzyme concentrations (same concentrations and symbols as in Figure 3).

for these systems as detailed in 3.3 Table II also shows that the hemicelluloses hydrolyze slowly in the presence of cellulases of *T. viride*.

3.3. Initial Rate of Glucose Formation as a Function of Enzyme and Substrate Concentration. The cellulase system is known to involve the synergic action of three types of activities:[1] (i) endoglucanases that act randomly on the amorphous parts of cellulose chains to generate new chain ends; (ii) exoglucanases that liberate cellobiose by endwise action on the nonreducing chain ends of crystalline and amorphous cellulose; (iii) β-glucosidases that mainly hydrolyze cellobiose into glucose.

Let us adopt a simple reaction network in which the synergic action of endo- and exoglucanase to produce soluble cellobiose is represented in one step followed by the hydrolysis of cellobiose into glucose. E_1 represents the endo- and exoglucanose, E_2 represents the β-glucosidase,

Table I
Glucose Formed as a Function of Time in Enzymatic Hydrolysis (Short Reaction Time)

A. Experiments at Constant Enzyme Concentration (1 g L^{-1}) and Various Substrate Concentrations[a]

init amt of substrate, g L^{-1}	glucose formed,[b] g L^{-1}									
	untreated substrate					pretreated substrate				
	init potential glucose[c]	1 h	2 h	3 h	4 h	init potential glucose[c]	1 h	2 h	3 h	4 h
2.5	1.06	0.02	0.05	0.05	0.06	1.15	0.08	0.15	0.28	0.38
5.0	2.11	0.05	0.10	0.13	0.16	2.31	0.11	0.22	0.42	0.57
10	4.23	0.09	0.15	0.19	0.22	4.62	0.15	0.31	0.57	0.76
25	10.57	0.20	0.32	0.43	0.49	11.55	0.19	0.39	0.72	0.95
50	21.15	0.28	0.47	0.63	0.75	23.10	0.15	0.38	0.71	0.94

B. Experiments at Constant Substrate Concentration (25 g L^{-1}) and Various Enzyme Concentrations[d]

amt of enzyme, g L^{-1}	glucose formed,[e] g L^{-1}							
	untreated substrate				pretreated substrate			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	5 h
0.5	0.09	0.20	0.27	0.32	0.10	0.18	0.30	0.51
1.0	0.16	0.26	0.42	0.49	0.19	0.40	0.65	1.02
2.5	0.27	0.48	0.58	0.66	0.51	0.98	1.35	2.27
5.0	0.39	0.60	0.71	0.79	0.91	1.38	2.25	3.90

[a] Using the reciprocal of (10), K_A and $v_{max} = k_2 e_{1,0}$ can be calculated for the pretreated substrate: K_A = 2.340 g of glucose L^{-1} and v_{max} = 0.270 g of glucose L^{-1} h^{-1}. [b] Relative error in glucose determination was 7%. [c] Obtained by quantitative saccharification. [d] Initial potential glucose 10.6 g L^{-1} untreated substrate. Initial potential glucose 11.55 L^{-1} pretreated substrate. [e] Relative error in glucose determination was 7%.

Table II
Formation of Cellobiose, Hexoses, and Pentoses as a Function of Time for the Pretreated Substrate (ps) and Untreated Substrate (us)[a]

time, h	saccharide formed, g $L^{-1} \times 10^2$										
	cellobiose		glucose		mannose		arab-inose	galactose		xylose	
	ps	us	ps	us	ps	us	ps us	ps	us	ps	us
1	12	8.8	18	17	0.7	–	0.8	0.9	0.4	4.7	1.2
3	24	8.6	49	38	1.8	0.6	1.1	1.6	1.4	5.8	1.4
24	88	5.4	294	104	14	4.5	1.1	8.7	4.7	11	3.0
48	97	4.8	461	137	34	11	1.6	23	11	21	5.2
144	80	2.8	790	182	74	34	3.5	37	24	34	10

[a] Substrate: 25 g L^{-1} enzyme: 1 g L^{-1}. These experiments were performed to correlate the rates of formation of the hydrolysis products. They cannot be compared with those given in the other tables and figures, the enzyme activity and experimental conditions being slightly different.

C and G are soluble cellobiose and glucose molecules, S is a vacant site on the substrate, and E_1S and E_2C are enzyme–substrate complexes. Then

$$E_1 + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1S \quad (1)$$

$$E_1S \xrightarrow{k_2} E_1 + C \quad (2)$$

$$E_2 + C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_2C \quad (3)$$

$$E_2C \xrightarrow{k_4} E_2 + G \quad (4)$$

Let us represent the number of E_1, E_2, S, C, G, E_1S, and E_2C per unit volume by e_1, e_2, s, c, g, (e_1s), and (e_2c). The adsorption equilibrium on the substrate is characterized by

$$K_A = k_{-1}/k_1 = e_1 s/(e_1 s) \quad (5)$$

If the quasi-steady-state approximation is valid

$$dc/dt = 0 = k_2(e_1s) + k_{-3}(e_2c) - k_3 e_2 c$$

and

$$d(e_2c)/dt = 0 = k_3 e_2 c - k_{-3}(e_2c) - k_4(e_2c)$$

with the consequence that $k_4(e_2c) = k_2(e_1s)$. This gives for the rate of glucose formation

$$dg/dt = k_4(e_2c) = k_2(e_1s) \quad (6)$$

If s_0 and e_0 are the total number of vacant sites and enzymes molecules per unit volume

$$s_0 = s + (e_1s) \quad \text{at low conversion} \quad (7)$$

$$e_{1,0} = e_1 + (e_1s) \quad (8)$$

Replacing e_1 in (5) by its value obtained from (8) gives

$$(e_1s) = e_{1,0}s/(K_A + s) \quad (9)$$

If, furthermore, $s_0 \gg e_{1,0}$, $s \simeq s_0$ at low conversion, and

$$v = \frac{-ds}{dt} = \frac{dg}{dt} = k_2(e_1s) = k_2\frac{e_{1,0}s_0}{K_A + s_0} \quad (10)$$

If this kinetic scheme, leading to the well-known Michaelis type equation, is obeyed, a linear relation must exist between the initial rate of hydrolysis and enzyme concentration on the one hand and between the reciprocal of the initial rate and the reciprocal of substrate concentration on the other hand. The parameter s is assumed to be proportional to the quantity of residual potential glucose in the sample, and s_0, the initial potential glucose, is determined by quantitative saccharification. The initial rate of reaction and its reciprocal calculated from Table I are given in Figures 5 and 6 for the pretreated and untreated substrates. Figure 5 (▲), Figure 6 (▲), and Figure 7 (▲) show that relation 10 is valid for the pretreated substrate.

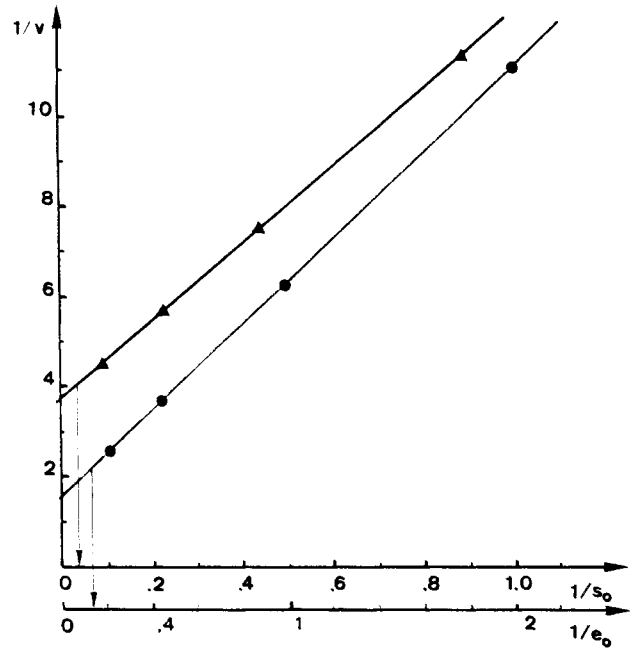

Figure 5. (▲) $1/v$ as a function of $1/s_0$ for the pretreated substrate, enzyme concentration: 1 g L^{-1}. (●) $1/v$ as a function of $1/e_0$ for the untreated substrate, initial wood concentration: 25 g L^{-1}. s_0 and e_0 in g L^{-1}, v in g L^{-1} h^{-1}. s_0 and e_0 in g L^{-1}, v in g L^{-1} h^{-1}. s_0 is the potential glucose obtained by quantitative saccharification.

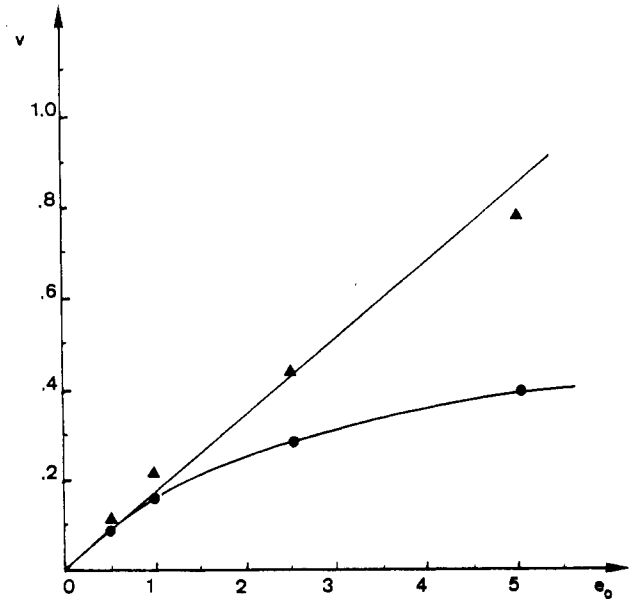

Figure 6. Initial rate of glucose formation as a function of enzyme concentration: (▲) pretreated substrate; (●) untreated substrate. Same units as in Figure 5, initial wood concentration: 25 g L^{-1}.

On the contrary, Figure 6 (●) indicates that the results obtained for the untreated substrate cannot be interpreted by eq 10. These results suggests use of an equation of the type

$$v = k_2 s_0 e_{1,0}/(K_A + e_{1,0}) \quad (11)$$

In this case, a linear relation must exist between the initial rate of hydrolysis and substrate concentration on the one hand and between the reciprocal of the initial rate and the reciprocal of enzyme concentration on the other hand. Figure 5 (●) and Figure 7 (●) indicate that relation 11 is valid for the untreated substrate, and Figure 7 (▲) shows that it is not obeyed by the pretreated one. Equation 11 has been proposed by Ollis[14] for enzymatic reactions of

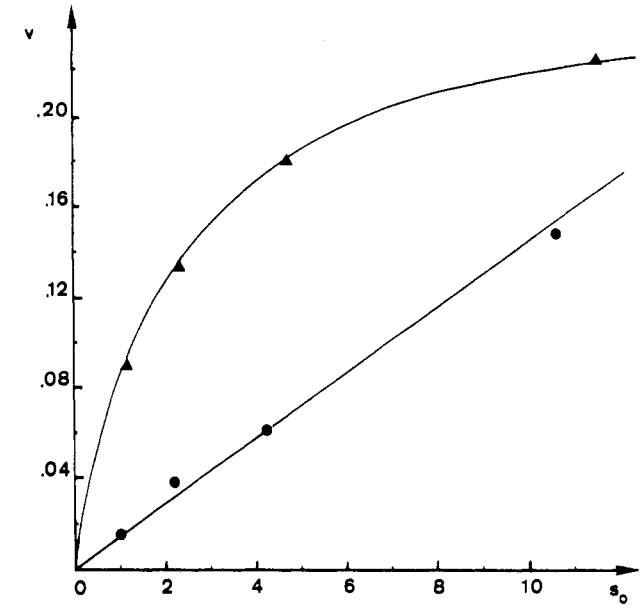

Figure 7. Initial rate of glucose formation as a function of substrate concentration: (▲) pretreated substrate; (●) untreated substrate. Same units as in Figure 5, enzyme concentration: 1 g L^{-1}.

heterogeneous substrates when the quantity of enzyme molecules is much larger than the number of vacant sites on the surface of the substrate. With the same kinetic scheme as before (eq 1–4), this equation is valid at low conversion if $e_{1,0} \gg s_0$.

Indeed, replacing s in (5) by its value obtained from (7) gives

$$(e_1 s) = e_1 s_0/(K_A + e_1) \quad (12)$$

Using (5) and introducing $e_1 = e_{1,0}$ if $e_{1,0} \gg s_0$ give (11).

The validity of eq 10 and 11 respectively for pretreated and untreated substrates suggests that the main effect of the pretreatment is to increase the number of active sites on the substrate and thus its accessibility to enzymes.

The kinetics of the hydrolysis of pretreated and untreated lignocellulosic materials have to our knowledge never been reported, but pure cellulose has been studied and reviewed recently.[1,7,8] Significant examples from such studies are given in ref 7–13. A simplified model including eq 1–3 is often used, the reaction product being considered to be reducing sugars as a whole. A Michaelis–Menten type equation was generally found to be valid for the initial reaction rate, in agreement with the present work. Two-substrate or multisubstrate sysytems have sometimes been introduced to take into account the different reactivity of the crystalline phase.

3.4. Glucose Formation at High Conversion. Inhibition. Figures 1–4 show that the limiting yield is almost 100% for the pretreated substrate, while it is very low for the untreated one. Several factors could be responsible for the difference between the two substrates. One, which is strongly suggested by the preceding section, is the limited accessibility of enzymes to the untreated substrates. The low limiting yield from the untreated substrate could reflect transformation into glucose of all the available accessible sites. It could also be due to enzyme deactivation by formation of complexes with soluble inhibitors or by adsorption on lignin sites of the substrate. Inhibition by product need not be considered when pretreated and untreated substrates are compared since it would be the same for both.

The possibility of irreversible adsorption on the substrate is considered first. The results of experiments are

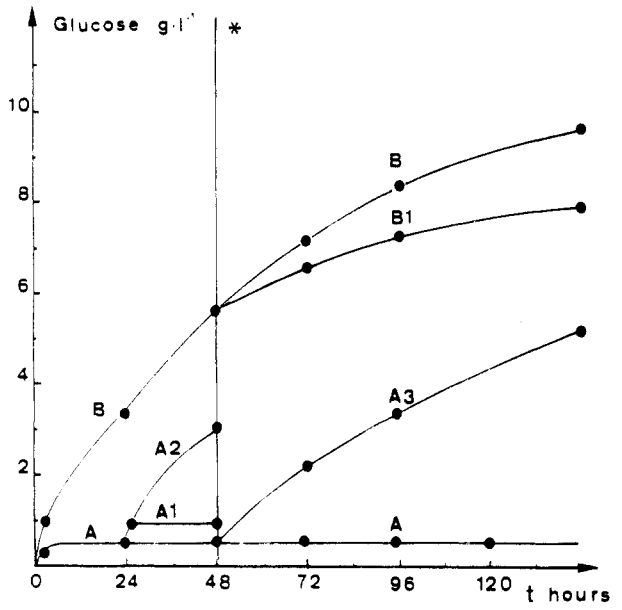

Figure 8. (A) Hydrolysis of untreated spruce wood in standard conditions; (A_1) addition of 250 mg of untreated substrate after 24 h; (A_2) addition of 250 mg of pretreated substrate after 24 h; (A_3) filtration after 48 h and addition of 250 mg of pretreated substrate; (B) hydrolysis of pretreated spruce wood under standard conditions; (B_1) filtration after 48 h and addition of 250 mg of pretreated substrate.

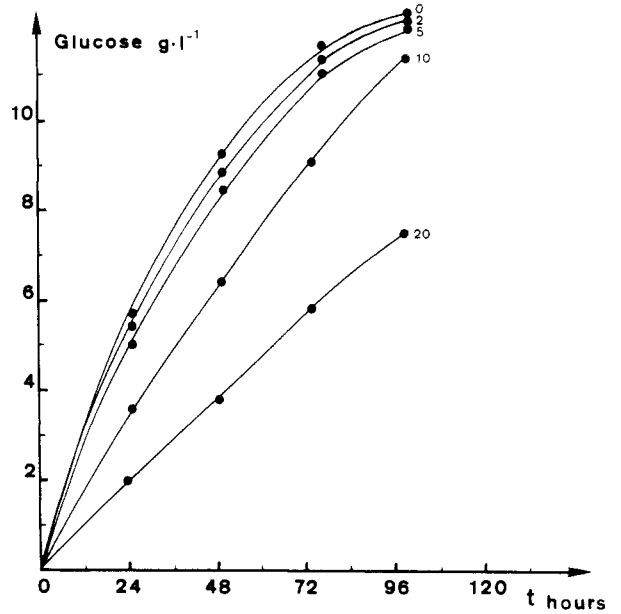

Figure 9. Enzymatic hydrolysis of pretreated substrate under standard conditions at different initial concentrations of glucose (g L^{-1}).

summarized in Figure 8. Two samples of untreated substrate were hydrolyzed under standard conditions (250 mg of substrate and 10 mg of enzyme in 10 mL of buffer) for 24 h (curve A). At this time, 250 mg of untreated (curve A_1) or pretreated (curve A_2) sustrate was added to each of the two samples. Comparison of curve A_1 with curve A and of curve A_2 with curve B, a reference curve for pretreated substrate hydrolyzed under the same standard conditions, shows that there was no significant loss in enzyme activity due to irreversible adsorption on the untreated substrate.

Reversible adsorption on the untreated substrate could however explain the preceding results. It was show not to occur by the following experiments. Untreated substrate was hydrolyzed under standard conditions. After 48 h, the reaction mixture was filtered to eliminate unreacted substrate and any adsorbed enzyme. Fresh pretreated substrate was then added to the filtrate (curve A_3). No significant inhibition is observed when curve A_3 is compared with the reference curve B for the pretreated substrate. This result proves that the low yield of glucose obtained from the untreated substrate does not result from either reversible or irreversible enzyme adsorption on lignin sites of this substrate. Some adsorption nevertheless occurs on the cellulose sites of the pretreated substrate, as has been reported in the literature for recovering enzymes. Indeed, if 250 mg of pretreated substrate is hydrolyzed under standard conditions, the mixture filtered after 48 h, and the filtrate incubated with 250 mg of fresh pretreated substrate, the rate of glucose formation is lower (curve B_1) than that of the reference sample (curve B). Exhaustion of the low number of accessible cellulose sites is thus the major cause of the limiting yield of glucose from the untreated substrate. For the pretreated substrate inhibition by product and limited accessibility both have to be considered to explain the lowering of the rate of reaction at intermediate and high conversions and the limiting yield of glucose.

Inhibition by glucose is now considered. Figure 9 gives the quantity of glucose formed as a function of time for different initial concentrations of glucose, the substrate and enzyme concentrations being the same in all experiments (respectively 25 and 1 g L^{-1}). Inhibition by glucose is shown to become perceptible for glucose concentrations exceeding about 5 g L^{-1}, which corresponds to a high conversion to glucose (about 50% of the potential glucose present in the sample). Allosteric inhibition has this characteristic and could be involved. The mechanism of inhibition by glucose was not investigated further. Inhibition by glucose has been studied in the enzymatic hydrolysis of pure cellulose and shown to occur at the same concentration as in the present work.[7-13] It is generally thought to involve competitive inhibition, although Howell and Stuck[10] have proposed a model involving noncompetitive inhibition.

The complete glucose–time curves for the pretreated substrate (Figures 1 and 3) are tentatively interpreted by using inhibition by glucose and the integrated form of eq 10

$$v_{max}t = s_0 - s + K_m \ln (s_0/s) \qquad (13)$$

From the experimental results obtained at constant enzyme concentration and given in Figure 1, theoretical glucose–time curves can be calculated by introducing in (13) the values of K_m and $v_{max} = k_2e_0$ determined with Table I and eq 10. An additional assumption is that s is proportional to the quantity of residual potential glucose in the sample and that s_0, the initial potential glucose, is given by the limiting yield obtained experimentally. The theoretical and experimental curves are compared in Figure 10. Excellent agreement between calculated and experimental curves is obtained at low substrate concentrations (curves 1–3). This agreement shows that limited accessibility is responsible for the limiting yield of glucose at low initial substrate concentrations. At higher initial substrate concentrations (curve 4), departure from the calculated curves occurs when the glucose concentration exceeds 5 g L^{-1}, as expected from the inhibition experiments given in Figure 9.

From the experiments performed at different initial enzyme concentrations (Figure 11), theoretical curves can be calculated with (13), the same value of K_m and v_{max} as in the preceding case for curve 2, and the assumption that

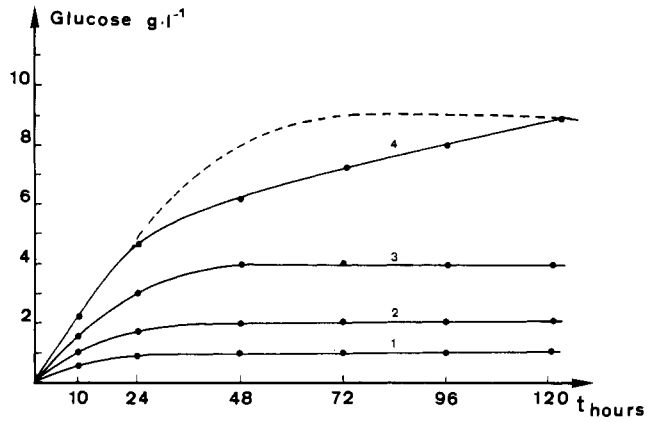

Figure 10. Enzymatic hydrolysis of pretreated spruce wood at different initial substrate concentrations (same symbols as in Figure 1): (—) experimental curves; (– – –) calculated curves.

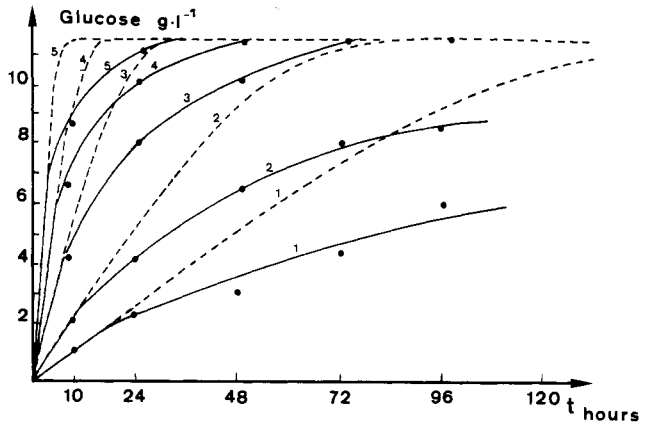

Figure 11. Enzymatic hydrolysis of pretreated spruce wood at different initial enzyme concentrations (same symbols as in Figure 3). (—) experimental curves; (– – –) calculated curves.

v_{max} is proportional to the initial enzyme concentration. This gives v_{max} values of 0.135, 0.675, and 1.350 g of glucose L^{-1} h^{-1} respectively for curves 1, 3, and 4. The agreement between calculated and experimental curves is good at low conversion, indicating that v_{max} is indeed proportional to e_0. At higher conversions however discrepancies occur between calculated and experimental curves. These discrepancies appear at lower conversions for lower e_0.

4. Conclusion

Pretreatment of spruce wood sawdust by sodium hypochlorite changes the kinetics of the enzymatic hydrolysis of the cellulosic part of the substrate into glucose. A simple reaction scheme involving one heterogeneous and one homogeneous enzymatic reaction and a steady-state approximation explains the experimental results assuming that the number of enzyme molecules per unit volume is larger than the number of vacant sites on the substrate for the untreated substrate and is smaller for the pretreated substrate. Pretreatment thus causes a significant increase in the number of vacant sites on the substrate. The low limiting yield of glucose obtained from the untreated substrate results from exhaustion of these vacant sites and not from inactivation of the enzyme by adsorption on lignin sites of the substrate.

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