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Influence of Fine Grinding on the Hydrolysis of Cellulosic Materials—Acid Vs. Enzymatic

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The relative influence of ball milling for various time periods on the course of enzymatic and dilute acid hydrolysis was investigated. The response of enzymatic hydrolysis to the extent of milling was quite dramatic. Cotton linters pulp was totally hydrolyzed in 10 days with 60 min of milling. The carbohydrates of red oak were 93% converted to sugar in the same time after 240 min of milling. It appears that, with sufficient milling, the carbohydrates of the three lignocelluloses investigated can be made almost totally accessible to enzymatic hydrolysis. Vibratory milling also yields substantial increases in the rates of dilute acid hydrolysis of all four substrates, nearly nine-fold for the cotton linters pulp and about five-fold for the three lignocelluloses. Thus vibratory milling represents an experimentally effective pretreatment.

Lignin and cellulose crystallinity are major deterrents to the chemical, enzymatic, and microbiological conversion of lignocellulosic residues to useful products. Lignin restricts enzymatic and microbiological access to the cellulose. Crystallinity restricts the rate of all three modes of attack on the cellulose. Thus if we are ever to make full use of the carbohydrate values contained in the many millions of tons of currently unused lignocellulosics generated in this country each year, some form of pretreatment must be employed to alter the fine structure of cellulose as well as disrupt or open up the lignin–carbohydrate complex.

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Of the many physical and chemical procedures thus far applied to enhance lignocellulose reactivity (1), fine grinding appears to offer the most direct response to both lignin and crystallinity. When the grinding is done in a vibratory ball mill, particle size can be reduced to micron dimensions, with attendant expansion of external surface area, and crystallinity can be essentially eliminated (2). This combination of events markedly influences carbohydrate accessibility and, hence, the degree of response of a milled lignocellulosic material to chemical, enzymatic, and microbiological attack.

The enhancing influence of fine grinding on enzymatic and microbiological attack has been amply demonstrated (3-11). Thus far, however, the relationship between fine grinding and chemical reactivity appears to have been neglected. This investigation was undertaken to fill this void by evaluating the influence of fine grinding on the kinetics of the saccharification process, both enzymatic and dilute acid. It is based on the responses derived from four cellulosic materials: cotton linters, newsprint, Douglas fir, and red oak.

Methods and Materials

Fine Grinding. Vibratory ball milling was done in the Forest Products Laboratory mill (5) using a sample charge of 10 g of dry material (48 hr at 40°C and 5 mm Hg) previously ground to pass a $^1/_2$-mm screen. The milling jar and its charge of 5 kg of $^1/_2$ in. (6.35 mm) polished and hardened steel balls was dried 4 hr under the same conditions and then cooled to room temperature in a desiccator. Milling times ranged from 0 to 240 min. Separation of the milled product from the balls was accomplished by mechanical agitation on a screened pan (Tyler Ro-Tap).

Cellulose Crystallinity. The cellulosic powders were pressed into pellets at 30,000 psi. X-ray diffraction traces were obtained on a Rigaku Denki diffractometer using nickel-filtered copper radiation over the angular range 10° to 30°. Crystallite size of the cellulose in the materials that were not ball-milled was determined by measuring the width of the 002 reflections. The crystallinity index (CI) proposed by Segal et al. (12) was used as a measure of the degree of lateral order. These indices are expressed as a percentage. Subject to the validity of the two-phase model of cellulose, this percentage is often used as a rough indication of the percentage of crystalline cellulose.

Enzyme Hydrolysis. Hydrolyses were conducted by the method of Moore et al. (10). This involves a digestion of 0.2-g samples with 6 mL of buffered enzyme solution at 40°C with constant rotation to effect continuous mixing. After the prescribed time period, a suitable aliquot is diluted, and Nelson's modification of the Somogyi procedure (*13*) is used to determine reducing sugar content. Maximum sugar yield for each level of vibratory milling was established on the basis of five hydrolysis periods ranging from 0 to 16 days. Residual potential sugar contents of hydrolyzed residues were determined by a quantitative acid saccharification as described in the following section,

Dilute Acid Hydrolysis. Hydrolyses were carried out at 180°C in scaled glass ampoules charged with 25 mg of sample and 0.25 mL of 0.1*N* H_2SO_4 according to the procedures described by Millett and Goedken (*14*). Five hydrolysis times, ranging from 4 to 160 min, were used for each milled sample. The ampoules were then carefully opened and their contents quantitatively transferred to microgooch crucibles and washed with about 20 mL of water. The filtrates were collected in 100-mL volumetric flasks, diluted to volume, and analyzed for net sugar yield (*14*). Residual potential sugar content of the residues was determined by quantitative saccharification involving a primary hydrolysis with 0.5 mL of 72% H_2SO_4, a secondary hydrolysis with 4% H_2SO_4, neutralization with calcium carbonate, and colorimetric analysis for total reducing sugars (*14*). Initial potential sugar content of each of the milled samples prior to hydrolysis was determined in the same manner. The data thus obtained provided the basis for graphical estimation of rates of hydrolysis and maximum sugar yields for each of the various levels of milling given to the four cellulosic substrates.

Materials. The four cellulosic substrates used in this study were a high-purity, sheeted, cotton linters pulp obtained from the Buckeye Cellulose Corp., newsprint from one of our local daily papers, bark-free Douglas fir wood, and bark-free red oak wood. All materials were broken down in a Wiley mill by successive passages through a series of decreasing screen sizes, with the final test samples being that stock passing through a ½-mm mesh. As indicated above, 10-g portions of these ground samples were vacuum-oven-dried prior to vibratory ball milling.

Results and Discussion

Crystallinity. Diffraction traces of the cellulosic materials are presented in Figures 1-4. Table I lists the crystallinity indices obtained from the diffraction patterns. Also included in Table I are the crystallite sizes of the unmilled materials obtained from measurement of the half widths of the 002 reflections. The 002 reflection is noticeably sharper in the case of the cotton cellulose. Crystallite size measures 54 A for the cotton cellulose compared to an average of 29 A from the wood celluloses.

Table I. Crystallinity Indices of Ball-Milled Materials

Ball Milling Time (min)	*Cotton*		*Newsprint*		*Douglas Fir*		*Red Oak*	
	CI (%)	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*
0	79	54	55	31	48	29	38	27
10	43	—	37	—	26	—	24	—
30	14	—	10	—	~ 3	—	6	—
60	0	—	0	—	0	—	0	—
120	0	—	0	—	0	—	0	—
240	—	—	—	—	—	—	0	—

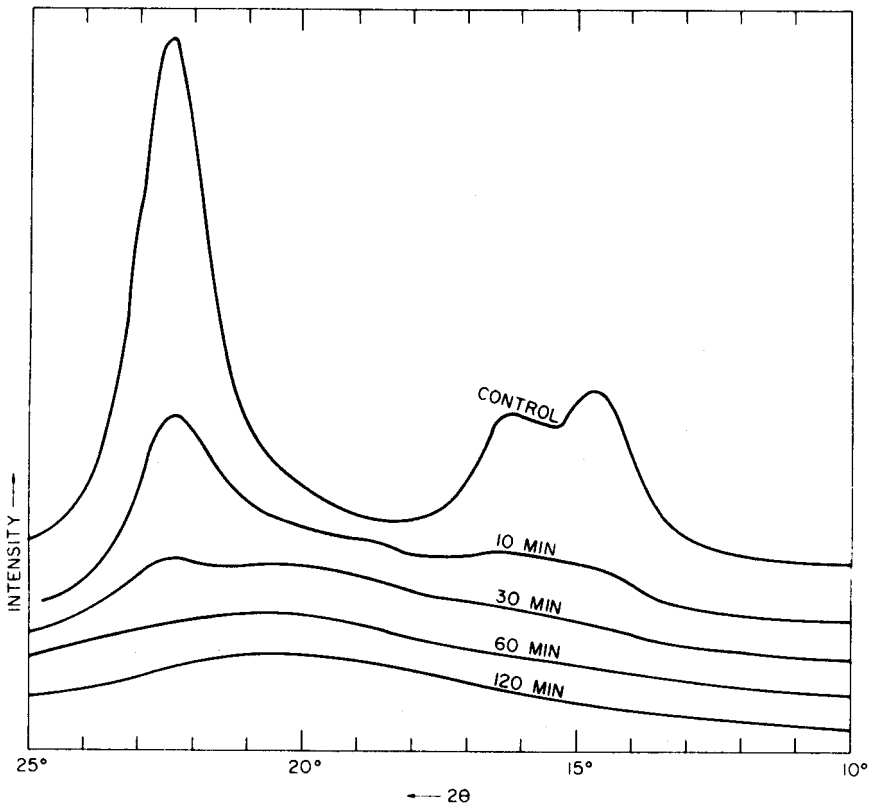

Figure 1. X-ray diffraction from cotton linters as affected by time of vibratory ball milling

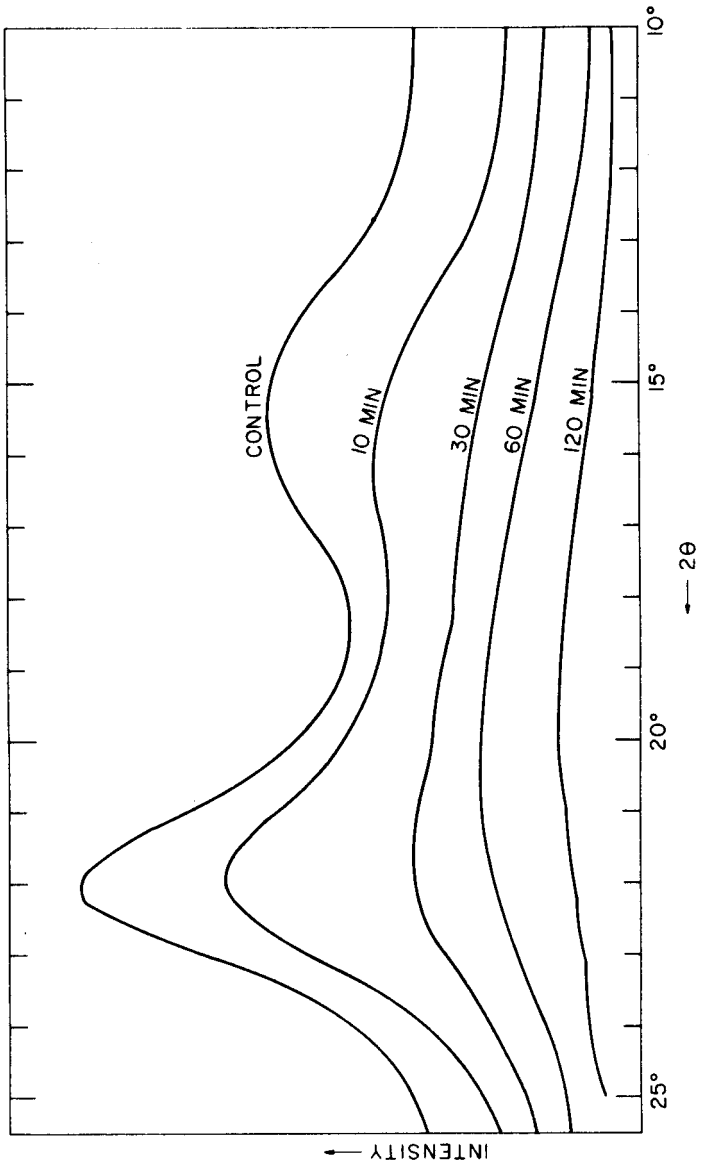

Figure 2. X-ray diffraction from newsprint as affected by time of vibratory ball milling

The effect of ball milling is similar in all cases. Within the first 10 min of milling, the 002 reflection is considerably broadened and appears slightly shifted to a smaller angle. This apparent shift is probably the result of the superposition of the broadened 002 peak upon the rising, broad, "amorphous" peak which centers about 18.5° (2θ). As ball milling continues, the crystalline characteristics decrease and the amorphous characteristics increase. After about 1 hr of milling time, the crystalline characteristics of all the cellulosic materials have disappeared.

The use of CI to represent the percentage of the crystalline component is unjustified among such a diversity of cellulosic materials, whose lignin components vary from 0 to about 30%. CI, as used here, is not intended to represent the proportion of the crystalline component. Rather, CI provides a means of quantifying the characteristics of the x-ray diffraction patterns. When applied to a set of similar samples, CI is a convenient measure of the degree of lateral order.

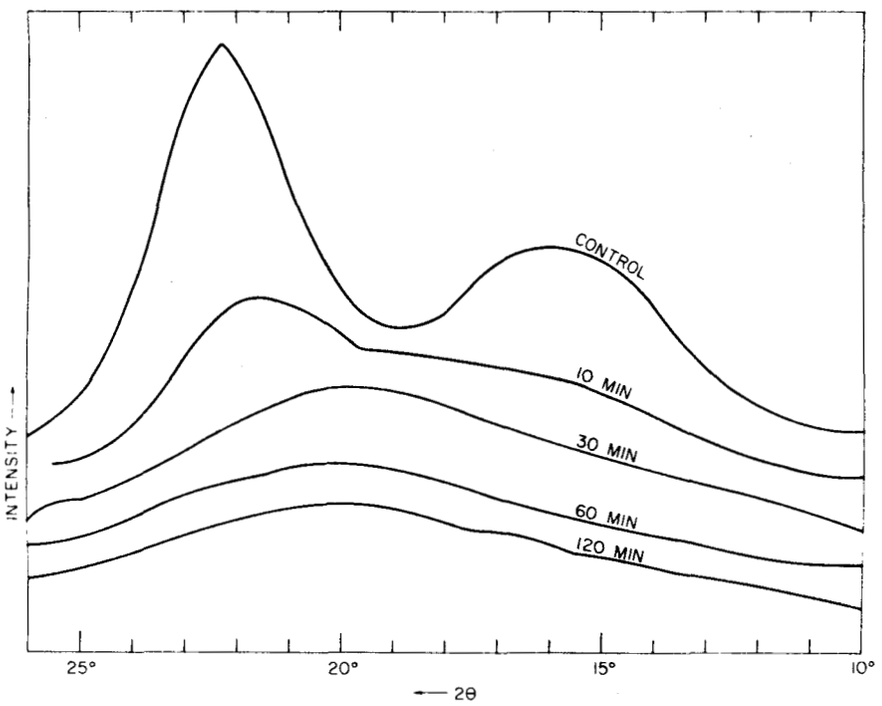

Figure 3. X-ray diffraction from Douglas fir as affected by time of vibratory ball milling

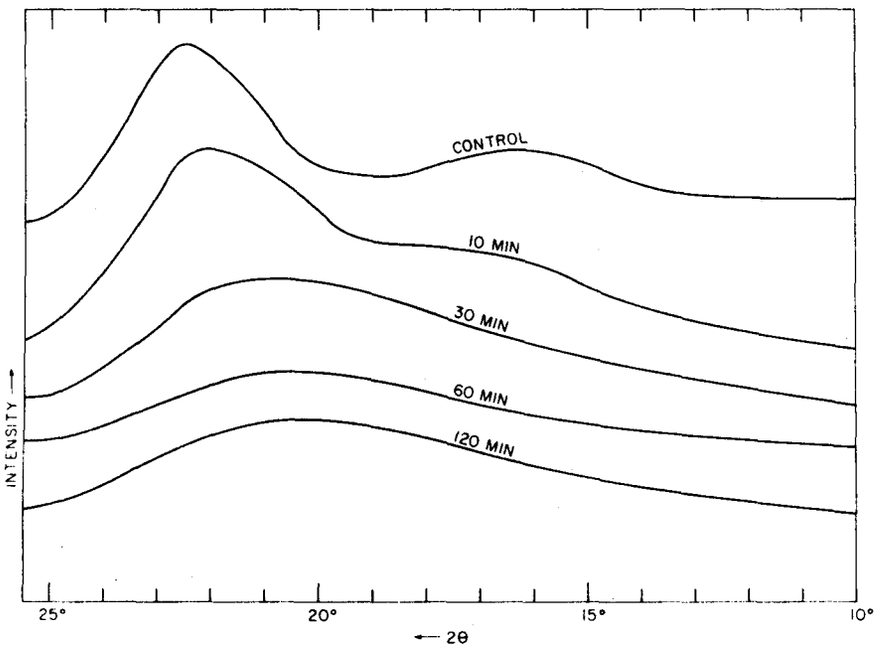

Figure 4. X-ray diffraction from red oak as affected by time of vibratory ball milling

Enzymatic Hydrolysis. All experimental data pertaining to the influence of fine grinding on the hydrolysis of the four cellulosic substrates are presented in Table II and plotted in the form of rate curves in Figures 5 and 6. The all-important roles played by cellulose accessibility and crystallinity in governing the course of enzymatic hydrolysis are readily apparent. With the unmilled starting materials, for example, approximately 10% of the total carbohydrates of Douglas fir and 6% of those for red oak are readily accessible and convertible to sugars in 2 days or less. Hydrolysis then ceases, probably due to complete shielding of the remaining carbohydrate by lignin. With cotton linters, hydrolysis proceeds at a moderate pace for the first 8 days of digestion and then slows down to an almost asymptotic approach to an 80% yield value (Figure 5). As suggested by Katz and Reese (*15*), this retardation in rate can well be the result of enzyme inactivation by hydrolysis products over the extended time periods used. Addition of fresh enzyme might have allowed hydrolysis to proceed to completion. In any event, total hydrolysis of a highly crystalline, lignin-free cellulose is a relatively slow process.

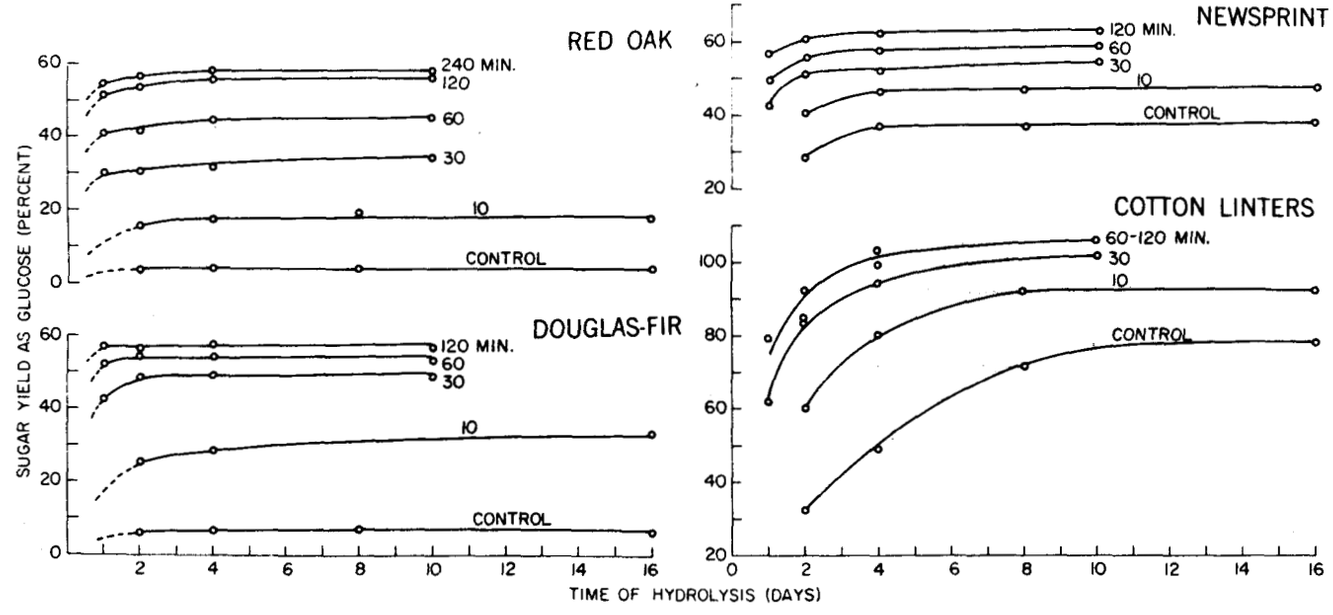

Figure 5. Effect of vibratory ball milling on the rate and extent of enzymatic hydrolysis

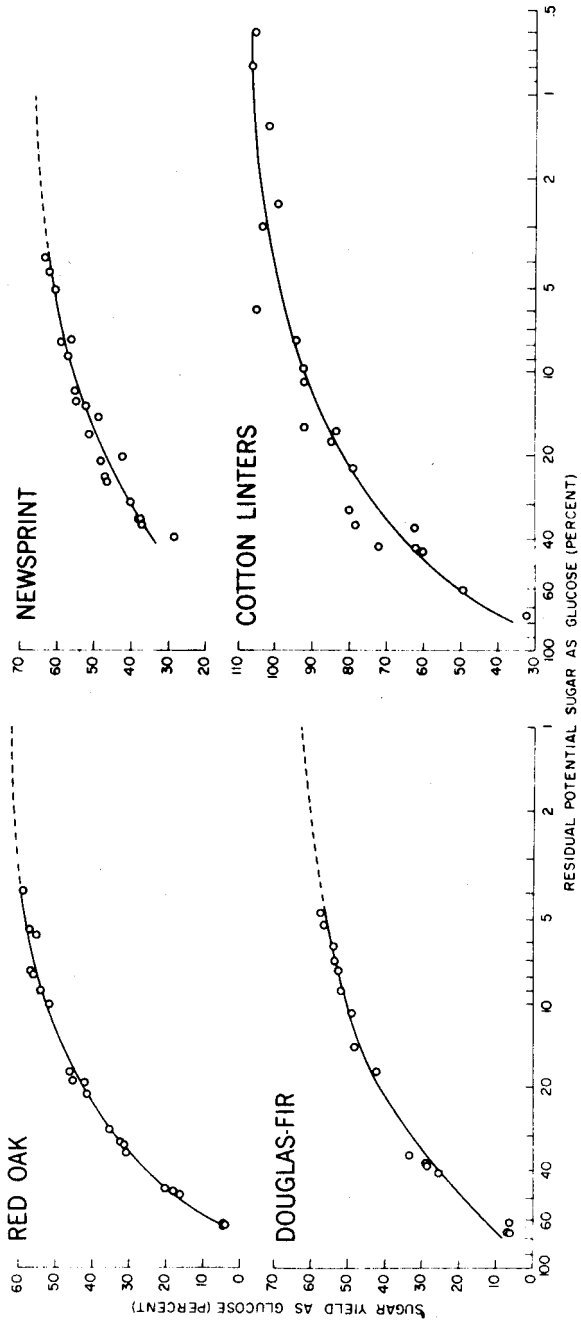

Figure 6. Effect of ball milling on sugar yield—enzymatic hydrolysis

Table II. Effect of Fine Grinding

Time of Milling (min)	*Time of Hydrolysis (days)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
0	0	—	—	—	—
	2	32.4	28.6	6.2	3.7
	4	49.3	37.1	6.7	4.0
	8	72.0	37.5	7.0	3.9
	16	78.5	38.7	6.3	4.0
10	0	—	—	—	—
	2	60.1	40.5	25.7	15.6
	4	80.1	46.8	28.8	17.3
	8	92.2	47.4	29.3	19.5
	16	92.4	48.4	33.6	17.3
30	0	—	—	—	—
	1	62.4	42.5	42.2	30.0
	2	85.2	51.2	48.5	30.4
	4	94.5	52.5	49.1	31.9
	10	102.0	55.1	48.9	34.9
60	0	—	—	—	—
	1	79.2	49.0	52.1	40.9
	2	92.4	55.4	53.9	41.5
	4	99.3	57.8	54.0	44.9
	10	106.7	59.4	52.9	45.6
120	0	—	—	—	—
	1	62.1	56.7	56.9	51.3
	2	83.5	60.9	56.7	53.7
	4	103.7	62.5	57.8	55.7
	10	105.4	63.9	56.9	56.6
240	0	—	—	—	—
	1	—	—	—	54.8
	2	—	—	—	56.5
	4	—	—	—	58.0
	10	—	—	—	58.3

[a] All listed values are averages of duplicate determinations.

Results with newsprint are roughly intermediate between those for wood and cotton linters. Hydrolysis of the delignified chemical pulp component (about 20% of the total newsprint furnish) and of the accessible carbohydrates of the groundwood component is accomplished with about 4 days of digestion. Further hydrolysis of the groundwood carbohydrate is then stymied by lignin as in the case of red oak and Douglas fir.

on Enzymatic Hydrolysis[a]

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
108.0	69.6	69.4	62.8
75.9	39.2	63.5	62.1
61.4	35.4	68.2	62.1
42.4	33.8	62.9	61.0
35.8	33.5	61.3	60.4
104.4	70.3	69.4	62.8
44.6	29.8	40.9	48.5
36.5	25.0	38.2	47.6
15.9	23.8	37.4	46.3
10.8	21.1	35.1	45.7
103.2	69.0	69.2	59.6
36.2	20.2	17.7	34.5
18.0	16.9	14.3	32.2
7.8	13.2	10.9	31.4
1.3	12.9	10.7	28.7
109.7	68.9	71.8	60.8
22.4	14.7	9.0	21.2
9.7	11.8	7.1	19.2
2.5	8.8	6.2	19.0
0.8	7.9	7.6	17.8
105.6	67.9	69.0	58.1
43.0	6.7	5.2	10.0
16.4	5.1	4.9	9.0
3.0	4.4	4.7	7.9
0.6	3.9	4.8	7.8
—	—	—	57.0
—	—	—	5.7
—	—	—	5.4
—	—	—	3.9
—	—	—	4.1

Vibratory milling enhances both the rate and extent of enzymatic hydrolysis, as shown by the families of curves in Figure 5. With sufficient milling, nearly theoretical yields of reducing sugars appear to be obtainable on all four substrates. This is best illustrated by the curves in Figure 6, which represent a composite of all of the data listed for each substrate in Table 11. The data in Table II show that, under the experimental

conditions employed in this study, cotton linters pulp is totally hydrolyzable with about 60 min of milling and 10 days of enzyme digestion. The carbohydrates of red oak are 93% convertible to sugar with 240 min of milling over the same digestion period. Both Douglas fir and newsprint have a milling requirement in excess of 120 min to attain close to their theoretical conversion limits.

Dilute Acid Hydrolysis. Data relative to the acid saccharification of the four substrates at various levels of vibratory ball milling are given in Table III and plotted as rate and yield curves in Figures 7 and 8. Table IV summarizes the half-life and maximum sugar yield values derived from these curves.

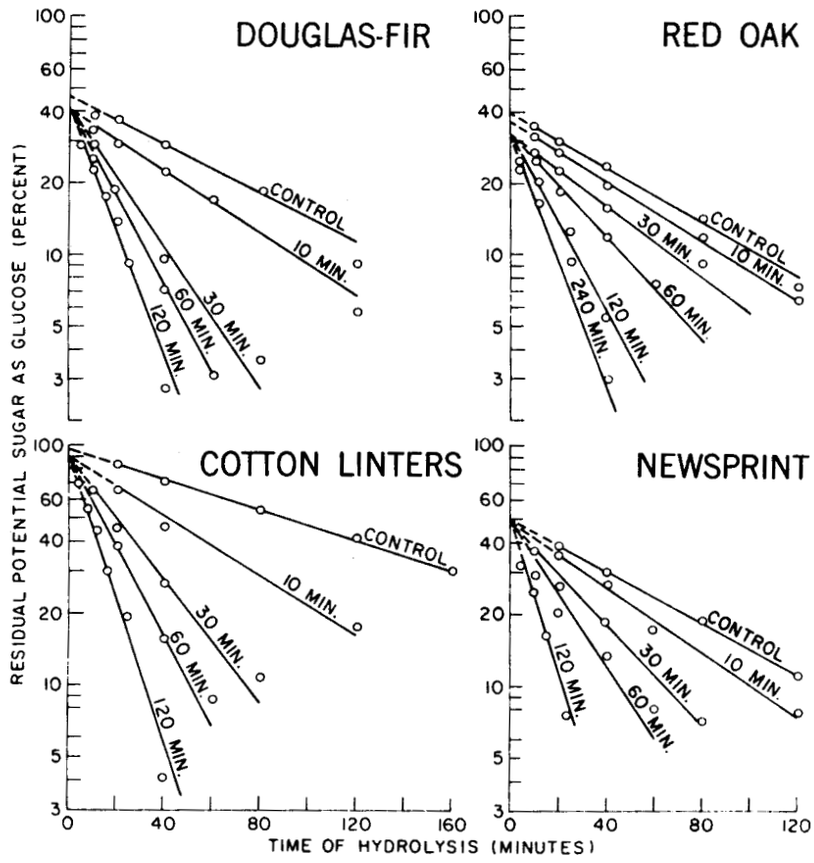

Figure 7. Effect of ball milling on rate of acid hydrolysis

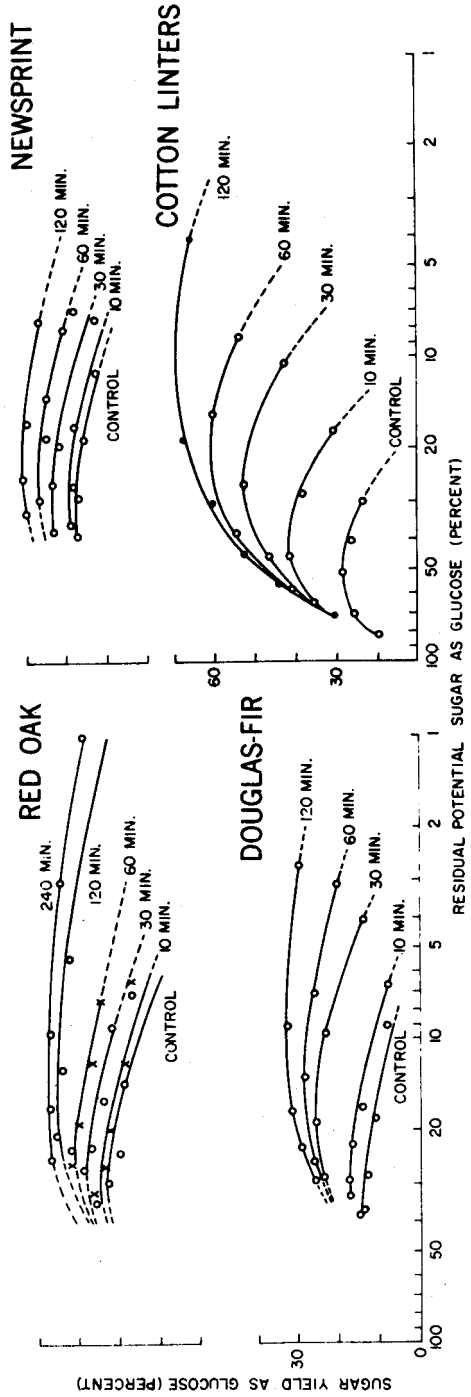

Figure 8. Effect of ball milling on sugar yield—acid hydrolysis

Table III. Effect of Fine Grinding on

Time of Milling (min)	*Time of Hydrolysis (min)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
0	0	—	—	—	0.6
	10	—	—	24.3	25.2
	20	19.7	17.4	28.4	22.2
	40	25.6	17.2	22.9	19.6
	80	28.5	15.4	21.0	18.4
	120	26.1	12.3	18.0	16.3
	160	23.4	—	—	—
10	0	—	—	—	1.0
	10	—	—	27.2	26.1
	20	35.4	18.6	27.2	23.7
	40	41.6	19.5	26.5	22.1
	60	—	18.0	23.9	—
	80	38.1	—	—	18.5
	120	30.5	12.4	18.1	16.3
30	0	—	—	—	1.2
	10	35.6	23.0	33.7	28.2
	20	46.3	23.2	35.6	26.2
	40	52.8	21.2	33.1	23.3
	80	42.5	18.6	24.2	21.1
60	0	—	—	—	1.1
	10	40.9	26.3	36.0	31.6
	20	54.5	24.8	38.3	29.7
	40	60.0	22.6	36.0	26.6
	60	53.2	20.4	30.8	24.4
120	0	—	—	—	1.1
	4	30.7	—	—	31.8
	5	—	29.6	35.7	—
	8	44.0	—	—	—
	10	—	30.7	39.0	—
	12	52.8	—	—	35.0
	15	—	29.5	41.2	—
	16	60.3	—	—	—
	25	67.5	26.4	42.5	33.4
	40	65.2	—	29.6	31.4
	60	—	—	—	25.2

Acid Hydrolysis (0.1*N* H_2SO_4 at 180°C)[a]

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
108.0	69.6	69.4	62.8
—	—	38.5	34.6
82.6	38.4	36.7	29.8
70.3	29.6	28.7	23.6
52.8	18.5	18.3	14.0
40.9	11.1	9.1	7.1
30.0	—	—	—
104.3	70.3	69.4	62.8
—	—	33.0	81.6
65.0	35.9	29.5	26.8
45.2	26.8	22.2	19.7
—	16.9	16.9	—
28.4	—	—	11.9
17.4	7.5	5.7	6.4
103.2	69.0	69.8	59.6
64.8	37.2	28.8	26.7
45.3	26.1	19.0	22.7
26.4	19.6	9.6	15.6
10.5	7.0	3.6	9.0
109.7	68.9	71.8	60.8
58.6	29.3	25.3	25.1
38.3	20.3	13.4	18.7
15.3	13.6	7.1	11.7
8.6	8.1	3.1	7.4
105.5	67.9	69.0	58.1
71.3	—	—	22.9
—	32.4	29.1	—
55.3	—	—	—
—	25.0	22.8	—
44.7	—	—	20.5
—	16.4	17.3	—
30.1	—	—	—
19.1	7.6	9.1	12.5
4.1	—	2.7	5.4
—	—	—	0.9

Table III.

Time of Milling (min)	*Time of Hydrolysis (min)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
240	0	—	—	—	1.0
	4	—	—	—	36.1
	5	—	—	—	—
	10	—	—	—	—
	12	—	—	—	36.5
	15	—	—	—	—
	25	—	—	—	36.2
	40	—	—	—	33.7
	60	—	—	—	27.1

[a] All listed values are averages of duplicate determinations.

Inspection of the data in Table IV shows that fine grinding causes a substantial increase in the rates of hydrolysis of all four substrates—nearly nine-fold for cotton linters and about five-fold for newsprint and Douglas fir after 120 min of milling. Red oak is somewhat less responsive, requiring 240 min of milling for a five-fold rate increase.

Maximum sugar yields exhibited a similar response to vibratory milling—a 2.4-fold increase in yield for cotton linters, a 1.8-fold increase for Douglas fir, a 1.7-fold increase for newsprint, and a 1.5-fold increase for red oak with 120 min of milling.

The batch hydrolysis conditions used in this work are not optimum for maximum production of sugar but were chosen many years ago at this laboratory for their sensitivity and convenience as a standard procedure for assessing the hydrolytic reactivity of a wide variety of cellulosic materials. Considerable improvement in maximum sugar yields is possible through the use of higher temperatures and acid concentrations in batch hydrolyses or through the use of percolation procedures whereby sugars are removed from the reaction zone as quickly as possible (*16, 17*). In no case, however, is it possible to conduct a dilute acid saccharification of a cellulosic material without some degree of sugar destruction. Thus maximum sugar yield obtained via dilute acid hydrolysis will always be somewhat lower than those attainable via enzymatic hydrolysis, even when both procedures are applied to highly reactive cellulose.

The effect of ball milling on the dilute acid hydrolysis of cellulose is similar in many ways to the effect electron irradiation has on enhancing saccharification (18). In the case of cotton linters, for example, maximum irradiation 10^8 rd) resulted in a half life of hydrolysis of about 7 min

Continued

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
—	—	—	57.0
—	—	—	24.6
—	—	—	—
—	—	—	—
—	—	—	16.6
—	—	—	—
—	—	—	9.4
—	—	—	3.0
—	—	—	0

Table IV. Effect of Vibratory Ball Milling on the Kinetics of Dilute Acid Hydrolysis[a]

Substrate	*Time of Milling (min)*	*Hydrolytic Half Life (min)*	*Maximum Sugar Yield (%)*
Cotton linters	0	95	29
	10	48	42
	30	23	53
	60	16	61
	120	11	69
Newsprint	0	56	18
	10	45	19
	30	29	24
	60	20	27
	120	10	31
Douglas fir	0	60	24
	10	47	27
	30	21	36
	60	16	39
	120	12	43
Red oak	0	53	23
	10	48	25
	30	40	28
	60	27	31
	120	16	35
	240	11	37

[a] 0.1*N* H_2SO_4 at 180°C.

and a maximum sugar yield of about 70%. Ball milling for 120 min provides a maximum sugar yield of 63% and a hydrolytic half life of 11 min (Table IV). Electron irradiation also enhances markedly the yield and rate of acid hydrolysis of a lignocellulosic, extracted sprucewood (*18*).

It is not clear, however, whether electron radiation would have beneficial effects upon the enzymatic hydrolysis of cellulose. It has been shown that the particle size reduction and surface area increase upon ball milling are probably more important than the reduction of crystallinity (19). Electron irradiation may not disrupt the shielding layer provided by the lignin to expose fresh cellulose to the action of large enzyme molecules.

Summary and Conclusions

The relative influence of vibratory milling on the course of enzymatic and dilute acid hydrolysis of four cellulosic substrates was investigated. The four substrates-cotton linters, newsprint, Douglas fir, and red oak—were vacuum-dried and then milled for various time periods ranging up to 240 min. Assays were then made of rate and extent of hydrolysis, maximum yield of reducing sugar, and cellulose crystallinity.

As anticipated, the response of enzymatic hydrolysis to extent of milling was quite dramatic, Under the assay conditions used, cotton linters pulp was totally hydrolyzed in 10 days with 60 min of milling. The carbohydrates of red oak were 93% convertible to sugar in the same period with 240 min of milling. It appears that, with sufficient milling, the carbohydrates of the three lignocelluloses are totally accessible to enzymatic attack. Thus when compared to the 6% maximum conversion of the original oak sawdust, vibratory milling does represent an experimentally feasible pretreatment.

Vibratory milling also yields substantial increases in the rates of dilute acid hydrolysis of all four substrates: nearly nine-fold for cotton linters and roughly five-fold for the three lignocelluloses. Increases in maximum sugar yields under simple batch conditions ranged from 60% to 140% over the yields for the unmilled materials.

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