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The influence of acetosolv pulping conditions on the enzymatic hydrolysis of Eucalyptus pulps

G. Vázquez, G. Antorrena, J. González, S. Freire, I. Crespo

Abstract *Eucalyptus globulus* wood was subjected first to HCl–catalysed delignification with 70% acetic acid under conditions realizing an incomplete $3 \times 3 \times 3$ factorial design (HCl concentration 0, 0.025 or 0.05%; temperature 120, 140 or 160 °C; reaction time 1, 2.5 or 4 h), and then to enzymatic hydrolysis. The hydrolysis kinetics conformed to both Ghose's empirical model and a biexponential equation. The biexponential fit implies the presence of both readily and reluctantly hydrolysed cellulose fractions, and the fitted coefficients show hydrolysis yield to depend largely on the digestibility of the latter. Multiple regression of performance variables on pulping conditions showed that neither the rate nor the extent of hydrolysis is greatest for pulps with minimum lignin or xylose contents; we attribute this circumstance to the condensation and precipitation of lignin under severe pulping conditions, which protects the cellulose of the pulp from enzymatic attack.

Introduction

Enzymatic hydrolysis of natural lignocellulosic materials is a heterogeneous reaction that often proceeds very slowly because enzymatic attack is hindered by the structural characteristics of the substrate. The chief relevant factors (Dunlap et al. 1976; Gharpuray et al. 1983) are lignin and hemicellulose content, surface area, pore size (Stone et al. 1969) and the crystallinity of the cellulose [digestibility decreases with increasing crystallinity; Walker and Wilson (1991)]. To increase the yield of enzymatic hydrolysis, the natural substrate must therefore be pretreated to break down cell walls, remove lignin, hemicellulose and lignohemicellulosic polymers, increase the surface area and pore size of the cellulose, and reduce crystallinity. Proposed pretreatments include physical, biological and chemical processes; the physical treatments mainly reduce crystallinity and increase surface area, while the chemical treatments mainly reduce lignin content.

That lignin shields enzyme–binding sites on the cellulose fibres of natural lignocellulosic materials is evidenced by the negative correlation between the lignin contents and enzymatic digestibilities of a wide range of such materials (Van Soest 1969). However, enzymatic digestibility does not always correlate positively with the extent of prior delignification of the substrate. The expected

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G. Vázquez (✉), G. Antorrena, J. González, S. Freire, I. Crespo
Department of Chemical Engineering, Faculty of Chemistry,
University of Santiago de Compostela, Avenida de las Ciencias s/n,
15706 Santiago de Compostela, Spain

positive correlation has been reported for hardwoods and softwoods delignified with acid chlorite (Sudo et al. 1976; Shimizu 1981) and for black cottonwood and aspen wood treated with methanol under acidic or neutral conditions (Chum et al. 1988), but no such correlation was found when a variety of organic solvents (amines or mono- or dihydric alcohols) were used to delignify corn stover, which afforded only very low glucose yields even when highly delignified (Lee et al. 1987).

The influence of hemicellulose content on the susceptibility of lignocellulosic materials to enzymatic hydrolysis is shown by the significantly increased hydrolysis yields that have been obtained after removal of hemicellulose (either selectively or together with lignin) with alkalis (Matsumara et al. 1977; Uçar 1990), dilute acids (Uçar 1990) or organic solvents (Chum et al. 1988). Removal of hemicellulose opens up the cellulose matrix and increases pore size (so allowing the enzyme greater access to the substrate), and alkali treatments further enhance these effects by causing the matrix to swell.

Since the enzymatic hydrolysis of lignocellulosic materials is a heterogeneous reaction, it does not exhibit Michaelis-Menten kinetics as do enzyme-catalysed reactions in solution (Lee and Fan 1983). The complexity of the reaction, which in practice involves a mixture of different cellulolytic enzymes rather than a single species, and in the course of which the lignocellulose structure and the accessibility of the cellulose change, has meant that most kinetic studies have used empirical or semi-empirical models (Lee et al. 1980; Ghose 1969; Chum et al. 1988; Biermann and McGinnis 1990; Van Dyke 1972), some of which incorporate structural parameters (Lee and Fan 1982; Fan et al. 1980; Gharpuray et al. 1983).

In a previous paper (Vázquez et al. 1995a), we reported how the lignin and potential glucose contents of pulp obtained by HCl-catalysed delignification of *Eucalyptus globulus* wood with acetic acid depend on the pulping conditions (HCl concentration, temperature and reaction time). In this paper, we report the kinetics of the enzymatic hydrolysis of the pulp, and the influence of the pulping conditions on the potential xylose content of the pulp (a measure of hemicellulose content) and on the rate and yield of the hydrolysis reaction.

Experimental

Delignification experiments were carried out in 150 ml Teflon-lined stainless steel reactors. In all cases, 70% acetic acid was used with a solid:liquid ratio of 1:10. The operating conditions for each experiment were selected in accordance with the incomplete $3 \times 3 \times 3$ experimental design (see below). For details of the pulping process and analytical methods, see Vázquez et al. (1995b, c).

Enzymatic hydrolysis

Enzymatic hydrolysis was performed with cellulases from *Trichoderma reesei* (Celluclast 1.5 L, from Novo) together with β-glucosidase from *Aspergillus niger* (Novozym 188, from Novo), the latter being included because the Celluclast cellulases are severely inhibited by cellobiose and some other hydrolysis products and have low cellobiase (β-glucosidase) activity. The operational conditions were: solid/liquid ratio 1/100; pH = 4.8 (0.05 N, citrate/citric acid buffer); temperature 50 °C; cellulase activity 0.3 FPU/ml and β-glucosidase activity 3.8 UI/ml; 0.01% formaldehyde as a biocide. For 48 h, these mixtures were magnetically stirred in 250 mL Erlenmeyer flasks partially submerged in a bath thermostatted. Samples of the reaction medium were removed periodically and filtered to remove unhydrolysed material, and their glucose content P (g glucose/g pulp) was

determined by the Somogyi-Nelson method; the remaining potential glucose content of the sample was calculated as $S = S_0 - P$, where S_0 is the potential glucose content of the starting pulp. Kinetic curves (P/S_0 or S/S_0 against time) were fitted to the data using a simplex algorithm.

Experimental design

The influence of the pulping conditions on the potential xylose content of the pulp and on the rate and yield of the hydrolysis reaction was studied using an incomplete $3 \times 3 \times 3$ factorial design (HCl concentration 0, 0.025 or 0.05%; temperature 120, 140 or 160 °C; reaction time 1, 2.5 or 4 h). Multiple regression analysis was used to fit polynomials of the form

$$Y_j = a_{0j} + a_{1j}X_1 + a_{2j}X_2 + a_{3j}X_3 + a_{12j}X_1X_2 + a_{13j}X_1X_3 + a_{23j}X_2X_3 + a_{11j}X_1^2 + a_{22j}X_2^2 + a_{33j}X_3^2$$

where Y_1 is the potential xylose content of the pulp (% oven-dried pulp); Y_2 is the value of P/S_0 after an arbitrarily chosen time (31 h); $Y_3 = k_2$, the rate constant of the major (slow) hydrolysis process (see Results and Discussion); and X_1, X_2 and X_3 are variables corresponding to pulping temperature, HCl concentration and pulping time respectively, but normalized to take values of −1, 0 and 1. The (X_1, X_2, X_3) sets used in the experimental design are listed in Table 1.

Results and discussion

Hydrolysis of unpulped wood

Not unexpectedly, enzymatic hydrolysis of unpulped *Eucalyptus globulus* wood gave poor results: the proportion of cellulose hydrolysed was 4.1% after 2 h, and this value was not markedly improved by a further 46 hours' hydrolysis. This

Table 1. Experimental and predicted values of Y_1 (potential xylose content in % oven-dried pulp), Y_2 (proportion of cellulose hydrolysed) and Y_3 [=k_2 of equation (2)] for eucalyptus wood pulp obtained under various conditions conforming to an incomplete $3 \times 3 \times 3$ factorial design (see text)

Run	X_1	X_2	X_3	Y_1		Y_2		$Y_3 \cdot 10^3$	
				Exp.	Predicted	Exp.	Predicted	Exp.	Predicted
1	−1	−1	0	15.63	15.85	0.27	0.25	6.86	6.10
2	−1	0	−1	15.85	16.37	0.30	0.31	9.00	9.16
3	−1	0	1	14.85	13.32	0.29	0.31	6.76	7.48
4	−1	1	0	7.98	8.77	0.34	0.33	9.31	9.18
5	0	−1	−1	14.10	13.36	0.28	0.29	7.10	7.69
6	0	−1	1	6.60	7.91	0.27	0.26	5.19	5.22
7	0	0	0	6.30	6.30	0.33	0.33	8.50	8.57
8	0	0	0	6.30	6.30	0.33	0.33	8.65	8.58
9	0	1	−1	9.25	7.94	0.33	0.33	8.32	8.28
10	0	1	1	2.40	3.14	0.22	0.21	4.83	4.24
11	1	−1	0	3.68	2.89	0.33	0.34	9.96	10.09
12	1	0	−1	5.93	7.46	0.43	0.40	12.16	11.44
13	1	0	1	0.81	0.29	0.24	0.24	6.77	6.61
14	1	1	0	0.00	−0.21	0.24	0.25	5.85	6.60

result confirms that for enzymatic hydrolysis to be viable, the wood must first undergo structural and/or compositional modification.

Effect of pulping conditions on potential xylose content

Increasing any of the controlled factors (especially temperature) appreciably decreased the xylan content of the pulp, as is illustrated in Fig. 1 for temperature and pulping time at a fixed HCl concentration of 0.025%. The coefficients of the regression equation obtained for potential xylose content are listed in Table 2. Pulping at the highest temperature (160 °C) for the longest time (4 h) almost completely removed xylans (Table 1).

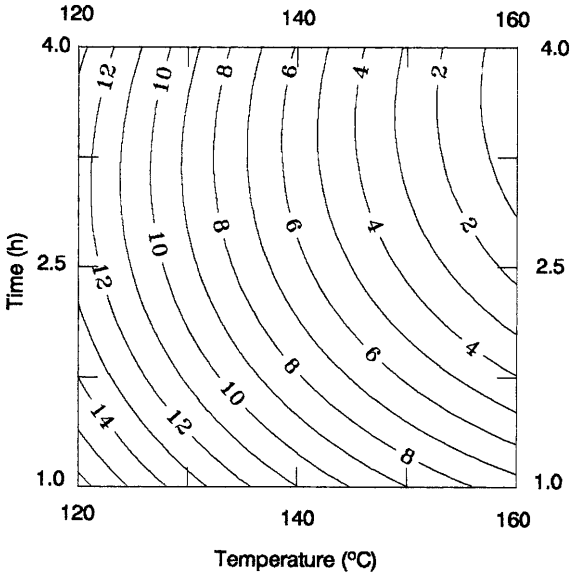

Fig. 1. Dependence of pulp potential xylose content (Y_1) on pulping temperature and time with an HCl concentration of 0.025%

Table 2. Coefficients of the regression equations fitted to the experimental data, with coefficients of determination, F values and the corresponding significance levels. Y_1, Y_2 and Y_3 as for Table 1

	Y_1	$Y_2 \cdot 10^2$	$Y_3 \cdot 10^3$
a_{oj}	6.30	33.40	8.58
a_{1j}	−5.49	0.45	0.35
a_{2j}	−2.55	−0.38	−0.10
a_{3j}	−2.56	−4.02	−1.63
a_{12j}	0.99	−4.08	−1.64
a_{13j}	−1.03	−4.19	−0.79
a_{23j}	0.16	−2.28	−0.40
a_{11j}	0.90	0.006	0.87
a_{22j}	−0.38	−3.94	−1.45
a_{33j}	2.16	−1.99	−0.77
R^2	0.9709	0.9229	0.9424
F-Ratio	14.82	5.32	7.27
Prob $F_{exp} > F_{tab}$	0.012	0.063	0.038

Effect of pulping conditions on the extent of hydrolysis

In the regression equation for the proportion of cellulose hydrolysed, the terms with the largest coefficients were the linear term in X_3, (corresponding to pulping time), the cross terms in X_1X_2 and X_1X_3 (corresponding to temperature × catalyst concentration and temperature × time), and the second degree term in X_2^2 (corresponding to catalyst concentration); see Table 2. With pulping mixtures containing 0.025% HCl, the proportion of cellulose subsequently hydrolysed was greatest for pulp obtained by a short treatment at high temperature, and least for lengthy high-temperature treatment (Fig. 2). In the absence of HCl, the position of the maximum was barely altered, but the altered position of the saddle shown in Fig. 2 meant that the position of the minimum (in the temperature–time region studied) now switched to short pulping times at low temperature. At the highest HCl concentration the minimum was located at the same position as for an HCl concentration of 0.025%, but the maximum was located at slightly longer pulping times and lower temperatures.

Thus in contrast to the findings of other authors (Sudo et al. 1976; Shimizu 1981; Chum et al. 1988), the proportion of cellulose hydrolysed was not negatively correlated with pulp lignin content or hemicellulose content. In fact, among the pulps obtained with non-zero HCl concentration, hydrolysis yield was minimum for the same pulps which had least xylan content (those obtained by long periods at high temperature). We attribute this lack of correlation to the condensation and reprecipitation of lignin that occurs under the most severe pulping conditions (Vázquez et al. 1995a, b); the reprecipitated lignin coats the surfaces of the pulp fibres, preventing the enzyme from accessing the cellulose. In this sense, the form in which lignin is present in the pulp sample is more important than its total lignin content.

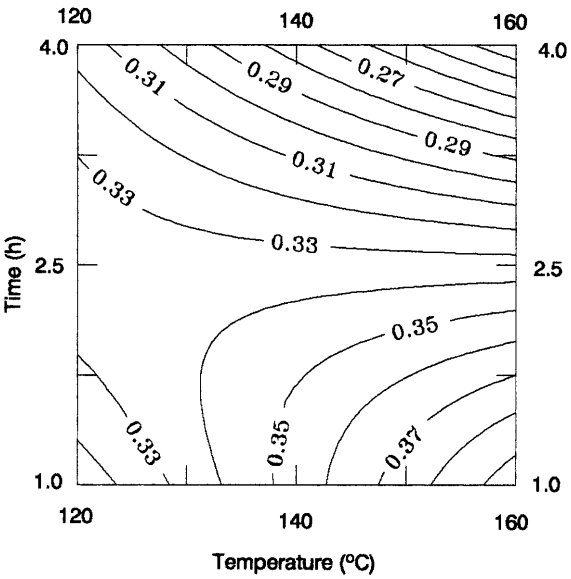

Fig. 2. Influence of pulping temperature and time on the percentage of cellulose hydrolysed by subsequent enzymatic reaction (Y_2), for pulps obtained with an HCl concentration of 0.025%

Hydrolysis kinetics

A rigorous study of the kinetics of the enzymatic hydrolysis reaction would necessitate construction of a mechanistic model including factors such as the structural parameters of the cellulose (in particular, surface area and crystallinity), the nature of the enzyme, and the characteristics of the heterogeneous cellulase/cellulose system as regards mass transfer, enzyme sorption processes and surface reactivities (Lee et al. 1980). In our study, the complexity of the model would be further increased by the need to take into account the structural and compositional changes in digestibility brought about by the various pulping treatments employed. However, several empirical or semi–empirical approaches have been developed which, while not taking all the above factors into account, nevertheless provide useful models of the progress of a part or of the entire hydrolysis process. The influence of pulping conditions on digestibility is adequately shown by the corresponding changes in the kinetic parameters of these models.

For each sample, the proportion of cellulose hydrolysed (P/S_0) was determined after reaction times ranging from 0–48 h, and these data were fitted with an empirical equation proposed by Ghose (1969):

$$\frac{P}{S_0} = kt^n \tag{1}$$

where t is reaction time in h, and n and k are fitted constants. Although Ghose (1969) considered this model to be suitable only for the initial phase of hydrolysis, it fitted our data well over the entire range of reaction times studied; Fig. 3 shows the results for three examples. For most samples, the calculated values of n were close to 0.5, in agreement with the Schutz-Arrhenius law for enzymatic hydrolysis reactions (Moelwyn-Hughes (1937) showed that this empirical law could be deduced by assuming diffusion control of the reaction rate).

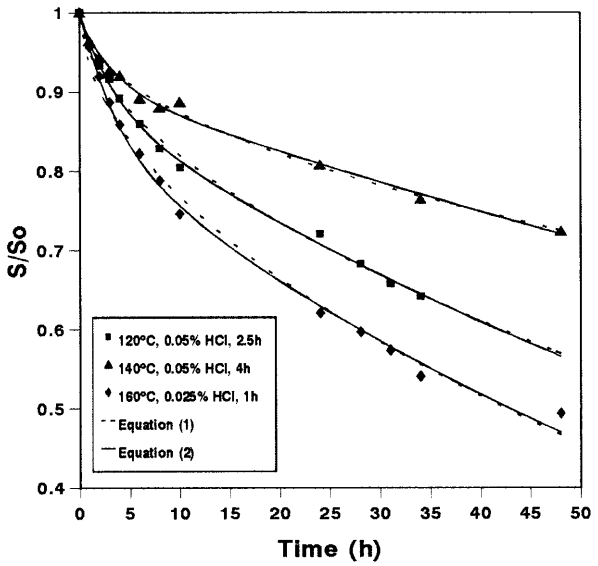

Fig. 3. Unhydrolysed cellulose (proportion) plotted against time for the enzymatic hydrolysis of eucalyptus pulps, with the results of fitting equations (1) and (2)

In spite of the good fit of equation (1), plots of log S/S_0 against time showed two clearly different regions reflecting the successive predominance of fast and slow reactions attributable to the hydrolysis of respectively an easily hydrolysed and resistant cellulose fraction. Similar behaviour has been reported for the hydrolysis of lignocellulosic materials by dilute acids (Saeman 1945), and for the enzymatic hydrolysis of cellulose (Van Dyke 1972) or of wood pulp obtained using organic solvents (Chum et al. 1988). In view of this finding, we fitted our data with an equation for two simultaneous pseudo first order reactions:

$$\frac{S}{S_0} = C_1 \exp(-k_1 t) + C_2 \exp(-k_2 t) \qquad (2)$$

where C_1 and C_2 are the proportions of easily hydrolysed and resistant cellulose respectively, and k_1 and k_2 are the rate constants (h^{-1}) for the corresponding hydrolysis processes. Following initial optimization of all these constants, they were refined, on the assumption that k_1 ought not to have been affected by our pulping conditions, by refitting Equation (2) with k_1 fixed at its mean value among the samples that contained the largest amounts of easily hydrolysed cellulose ($C_1 > 0.11$), whose initially determined values of k_1 were presumed to be the most accurate. The value of k_1 so fixed was 0.283 h^{-1}, and the final values of C_1, C_2 and k_2 (which actually differed little from the values first fitted) are listed in Table 3. With these constants, equation (2) fitted the data well over the entire range of reaction times studied, giving predictions of S/S_0 very similar to those afforded by equation (1).

Since the proportion of easily hydrolysed cellulose (C_1) was in all cases small (0.076–0.164), the total proportion of cellulose hydrolysed depended mainly on the tractability of the resistant fraction. In keeping with this conclusion, regression analysis of the influence of the pulping conditions on the rate constant k_2 (=Y_3) showed (Tables 1 and 2) that the regression terms most affecting Y_3 are those in X_3, X_1X_2, X_1X_3 and X_2^2, i.e. the same terms (and with the same signs) as most influence Y_2, the proportion of cellulose hydrolysed. The contour

Table 3. Optimized parameters obtained upon fitting equation (2) to the kinetic data for the hydrolysis of eucalyptus wood pulp with k_1 fixed at a value of 0.283 h^{-1}

Run	C_1	C_2	$k_2 \cdot 10^3$ (h^{-1})
1	0.089	0.904	6.86
2	0.092	0.906	9.00
3	0.115	0.882	6.76
4	0.115	0.885	9.31
5	0.098	0.891	7.10
6	0.129	0.867	5.19
7	0.122	0.879	8.50
8	0.111	0.882	8.65
9	0.119	0.881	8.32
10	0.085	0.909	4.83
11	0.086	0.907	9.96
12	0.164	0.843	12.16
13	0.077	0.918	6.77
14	0.077	0.916	5.85

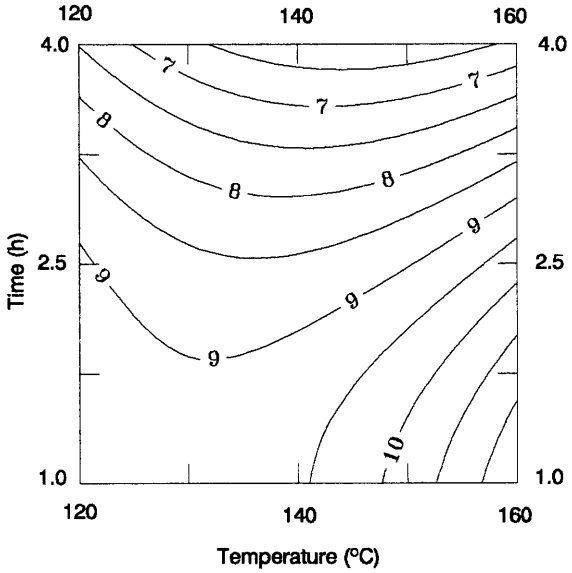

Fig. 4. Influence of pulping temperature and time on the rate of the slow hydrolysis process during subsequent enzymatic reaction (Y_3), for pulps obtained with an HCl concentration of 0.025%

diagrams showing the variation of Y_2 and Y_3 with pulping time and temperature for pulps obtained with an HCl concentration of 0.025% (Fig. 2, 4) are accordingly very similar. As noted above in discussing the proportion of cellulose hydrolysed, we attribute the variation in the recalcitrance of the resistant cellulose fraction chiefly to the condensation and deposition of lignin on pulp fibres during pulping, rather than to the structural characteristics of the cellulose itself.

Conclusions

Like other lignocellulosic materials, untreated *Eucalyptus globulus* wood is highly resistant to enzymatic hydrolysis, less than 5% of the cellulose fraction being hydrolysed after 48 hours' reaction. Our investigation, using an incomplete factorial design, of the influence of pulping conditions on the enzymatic digestibility and related characteristics of eucalyptus pulp obtained by an acetosolv process has shown that, contrary to what might have been expected in view of reports by other researchers, the proportion of cellulose hydrolysed is not negatively correlated with pulp lignin and/or xylan content. Rather, pulping conditions mainly affect the extent of hydrolysis by determining whether the lignin present in the pulp is largely lignin close to its native state or lignin which has undergone condensation and/or reprecipitation during pulping; high catalyst concentration, high temperature and long reaction times during pulping favour the latter processes and hence decrease the digestibility of the cellulose by reducing its accessibility for the enzyme.

Our data for the kinetics of the enzymatic hydrolysis reaction were satisfactorily fitted by Ghose's model. The values of n were in general close to 0.5, showing compliance with Schutz-Arrhenius empirical law for enzymatic hydrolysis, and thus suggesting that the rate of enzymatic hydrolysis is diffusion controlled. The data were also fitted well by a model for two simultaneous

first–order hydrolysis reactions with different rates, and polynomial regression of the rate constant for the slower on the pulping conditions yielded an equation very similar to that found for the proportion of cellulose hydrolysed, suggesting that overall yield is largely governed by the slow reaction.

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