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Effect of selected operational variables on the susceptibility of NaOH-pretreated pine wood to enzymatic hydrolysis: a mathematical approach

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Summary *Pinus pinaster* wood samples were treated with NaOH solutions in order to improve their susceptibility to enzymatic hydrolysis. The variations caused by pretreatments on the chemical composition of treated samples and on the extent of enzymatic saccharification were correlated with the experimental variables studied (temperature, liquor/wood ratio and alkali concentration). Empirical models were used to assess the extraction and the hydrolysis steps. The reliability of the mathematical models was confirmed by further experimentation. Additional aspects affecting the studied process (such as selectivity of extraction and kinetics of enzymatic hydrolysis) are discussed.

Introduction

Processes for biomass utilization based on the hydrolysis of the polysaccharide fraction by enzymes provide an interesting alternative to conventional chemical pulping, because of their ability to produce easily fermentable sugar solutions under mild experimental conditions.

Limited reaction rates and yields are reached when native lignocellulose is used as substrate for enzymatic hydrolysis. In order to limit these drawbacks, the chemical composition and/or the physicochemical features of the raw material must be altered. Removal of the lignin fraction (at least in part) favors the accessibility of enzymes to cellulose. The physicochemical features of substrate (crystallinity, polymerization index, available surface area, water holding capacity, and so on) are also main factors to be considered (Blanch and Wilke, 1983; Gharpuray et al. 1983 a and 1983 b).

Treatments with alkaline solutions have been proposed to enhance the hydrolysis of lignocellulose. Sodium hydroxide causes both partial delignification and swelling of substrates, leading to improved enzymatic saccharification yields (Gharpuray et al., 1983 a).

In the North-West of Spain, about 2.10^6 m^3 of *Pinus pinaster* wood are commercialized each year. Timber industries and particleaboard, plywood and pulp mills are the main destinations of this type of wood.

This work deals with the utilization of pretreated pine wood as substrate for enzymatic hydrolysis. An enzymatic complex (*Trichoderma reesei* cellusase), supplemented with β-glucosidase from *Aspergillus niger* to avoid accumulation of cellobiose in the reaction media, was used to perform the hydrolysis of NaOH-extracted pine wood. The effects of three selected operational variables affecting the pretreatment (temperature, liquor/wood ratio and sodium hydro-

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xide concentration) on four dependent variables (measuring the extraction yield, the chemical composition of treated samples and the extent of the enzymatic saccharification) were studied. An incomplete, factorial, centered experimental design was used to develop mathematical models giving empirical interrelationships between operational and experimental variables, which were used to assess the delignification and hydrolysis stages.

Experimental

Raw material

Samples of *Pinus pinaster* wood, collected in a local particleboard mill, were milled, screened to

select the fraction of particles with a size between 0.2 and 1 mm, homogenized (to ensure identical composition among the different aliquots taken from the wood lot), air-dried and stored.

Analysis of samples

The wood samples were analyzed for moisture, ash and extractives content (Browning, 1967; Vázquez et al., 1987 a). Aliquots of untreated wood were subjected to quantitative acid hydrolysis using 72% sulphuric acid following standard methods (Vázquez et al., 1987 b). Klason lignin was measured as the insoluble residue after treatment. Hydrolysates were neutralised and submitted to spectrophotometric and cromatographic determinations. Reducing sugars in hydrolysates were determined by the Somogyi-Nelson method, and the results (after stoichiometric correction) are expressed as percent of polysaccharides in wood. Glucose in hydrolysates was determined by HPLC using two Shodex SH-1 011 columns with 0.015 M sulphuric acid as mobile phase and refractive index detection. The results obtained are expressed (after stoichiometric correction) as glucan. So, this term include both cellulose and glucose polymers belonging to hemicelluloses.

Alkine treatment of wood

Wood samples and NaOH solutions were autoclaved for 3 h at 100–130 °C. The operational variables considered were: temperature, liquor/wood ratio and NaOH concentration of the extraction media. The solid residues from the different treatments were subjected to quantitative acid hydrolysis in the same way above described for native wood, in order to determine their contents in Klason lignin, polysaccharide and glucan.

Enzymatic hydrolysis

The enzymatic hydrolyses of pretreated wood were performed using cellulases from *Trichoderma reesei* (Celluclast, Novo, Denmark) and β-glucosidase from *Aspergillus niger* (Novozym, Novo, Denmark) (Vázquez et al., 1987 b). Enzymatic activities of commercial solutions were determined using reported methods (Mandels et al., 1976; Paquot et al., 1982). The operational conditions used for enzymatic hydrolyses were as follows: liquid/solid ratio = 20, temperature = 48.5 °C, reaction time = 48 h, pH = 4.85 (citric acid-citrate buffer 0.05 N), cellulolytic activity = 0.30 FPU/mL, β-glucosidase activity = 4.0 UI/mL. During the hydrolysis processes and at the end of reaction, samples were taken from the reaction medium at given time intervals, filtered through 0.45 μ membranes and analyzed for total and individual sugars by the spectrophotometric or HPLC methods as above. The data were fitted to an hyperbolic model, which allowed the estimation of both the highest conversion (corresponding to a reaction time = ∞) and the initial hydrolysis rate (see below).

Results and discussion

Experimental results and statistical analysis

Pretreatments in alkaline media were performed during 3 h. Three operational variables were selected to study the NaOH-treatment: temperature (T), liquor/wood ratio (LWR) and sodium hydroxide concentration (C). Their influence on four dependent variables (residue yield of ex-

Table 1. Variables considered in the study of alkaline extraction and enzymatic hydrolysis

Independent variables and variation ranges
T = temperature (100–130 °C)
LWR = liquor/wood ratio (6–10 g/g)
C = NaOH concentration (1–10 g NaOH/100 g solution)

Dependent variables
y_1 = residue yield, g residue recovered after alkaline treatment/100 g sample submitted to alkaline treatment, o. d. basis
y_2 = polysaccharide content of alkali-treated samples, g polysaccharides/100 g alkali-treated wood, o. d. basis
y_3 = glucan content of alkali-treated samples, g glucan/100 g alkali-treated wood, o. d. basis
y_4 = maximum[a] conversion of enzymatic hydrolysis, g sugars/100 g potential sugars[b]

[a] Maximum conversion: obtained by fitting the data concentration/time to an hyperbolic model
[b] Potential sugars: amount of sugars corresponding to the theoretical conversion of the polysaccharides contained in the substrate into monosaccharides

Table 2. Structure of the incomplete, centered, second-order experimental design used

Experiment	T [a]	LWR[a]	C[a]
1	100	6	5.5
2	100	8	1
3	100	8	10
4	100	10	5.5
5	115	6	1
6	115	6	10
7	115	8	5.5
8	115	8	5.5
9	115	8	5.5
10	115	10	1
11	115	10	10
12	130	6	5.5
13	130	8	1
14	130	8	10
15	130	10	5.5
16	115	10	5.5

[a] T, LWR and C as in Table 1

Table 3. Dimensionless independent variables

Variable	Definition
Dimensionless temperature, x_1	(T-115)/15
Dimensionless liquor/wood ratio, x_2	(LWR-8)/2
Dimensionless NaOH concentration, x_3	(C-5.5)/4.5

T, LWR and C as in Table 1

traction, polysaccharide content of treated samples, glucan content of treated samples, maximum yield of enzymatic hydrolysis) were determined under a variety of experimental conditions. Table 1 lists the independent variables and their variation ranges, as well as the dependent variables and their definitions.

The experiments performed (see Table 2) were selected according to an optimized incomplete, factorial, centered, second-order design (Poirier et al., 1987). The results obtained allowed the development of empirical models providing reliable information on the chemical alterations caused by extraction and on the saccharification step (Akhnazarova and Kafarov, 1982; Box et al., 1988). This methodology has been previously utilized in the study of chemical or biotechnological processing of lignocellulose (Poirier et al., 1987; David et al., 1988; Vázquez et al., 1992 a; Vázquez et al., 1992 b).

The independent variables shown in Table 1 must be converted into normalized, dimensionless ones before statistical calculations. The definitions of the dimensionless independent variables, having variation ranges (−1, 1), are shown in Table 3.

Table 4 lists the operational conditions used for experimentation (expressed using dimensionless variables) and the experimental results determined for the effects $y_1 \ldots y_4$. These data were fitted by multiple regression to models having the generalized form:

$$y_j = b_{oj} + \sum_{i=1}^{3} b_{ij} \cdot x_i + \sum_{i=1}^{3} \sum_{k=1}^{3} b_{ikj} \cdot x_i \cdot x_j$$

where:

y_j (j = 1 to 4) are the dependent variables defined in Table 1, x_i or x_k (i or k: 1 to 3, $k \geq i$) are the dimensionless independent variables defined in Table 3, and $b_{oj} \ldots b_{ikj}$ are the regression coefficients, calculated from the experimental data by the least-squares method.

Table 5 shows the set of coefficients obtained by regression, the statistical parameters used to measure both the correlation and the statistical significance of the models, and the significant coefficients at the 90 and 95% confidence level. It can be observed the high degree of sig-

Table 4. Operational conditions studied (expressed using dimensionless variables) and experimental results determined for the dependent variables

	Independent variables			Dependent variables			
Experim.	x_1	x_2	x_3	y_1	y_2	y_3	y_4
1	−1	−1	0	79.3	57.7	45.3	39.4
2	−1	0	−1	83.4	60.8	44.1	26.5
3	−1	0	1	79.7	62.4	48.6	33.1
4	−1	1	0	78.0	61.3	48.1	25.5
5	0	−1	−1	85.7	54.6	47.6	24.8
6	0	−1	1	75.7	64.5	50.9	47.2
7	0	0	0	75.8	60.8	49.9	31.7
8	0	0	0	76.3	61.4	48.5	33.7
9	0	0	0	75.7	61.4	47.6	31.1
10	0	1	−1	78.9	62.8	48.9	25.5
11	0	1	1	76.2	64.4	51.4	43.3
12	1	−1	0	73.8	62.2	52.7	35.7
13	1	0	−1	82.6	61.8	47.4	22.5
14	1	0	1	70.3	67.4	49.8	49.8
15	1	1	0	71.2	65.5	51.0	35.5
16	0	1	0	75.0	63.6	49.4	30.4

Table 5. Regression coefficients and statistical parameters

a) Coefficients

Coefficient	Variable (y_j)			
	y_1	y_2	y_3	y_4
b_{0j}	75.99	61.33	48.54	31.97
b_{1j}	−2.81	1.84	1.85	2.38
b_{2j}	−1.23	1.97	0.27	−2.31
b_{3j}	−3.59	2.34	1.59	9.26
b_{12j}	−0.33	−0.08	−1.13	3.43
b_{13j}	−2.15	1.00	−0.53	5.18
b_{23j}	1.83	−2.08	−0.20	−1.15
b_{11j}	−0.29	0.89	−0.70	−0.01
b_{22j}	−0.08	−0.45	1.34	1.92
b_{33j}	3.26	0.79	−0.27	1.16

b) Statistical parameters

Variable	R^2	Corrected R^2	F_{exp}[a]	Prob $[F_{exp} > F_{st}]$[a]
y_1	0.9886	0.9715	57.75	< 0.01
y_2	0.9670	0.9174	19.51	< 0.01
y_3	0.8928	0.7320	5.55	< 0.03
y_4	0.9690	0.9224	20.82	< 0.01

c) Significance of coefficients

Significant coefficients at the 95% confidence level:

b_{01}, b_{11}, b_{21}, b_{31}, b_{131}, b_{231}, b_{331}
b_{02}, b_{12}, b_{22}, b_{32}, b_{232}
b_{03}, b_{13}, b_{33}
b_{04}, b_{14}, b_{24}, b_{34}, b_{124}, b_{134}

Other significant coefficients at the 90% confidence level

b_{132}, b_{112}
b_{123}, b_{223}

[a] F_{exp} defined as the ratio between the mean squares of model and error. F_{st} defined as the statistical value of F for the degrees of freedom of model and error

nificance obtained for the models, on the basis of the results obtained by performing a "*F*" test. Only some of the contributions to the effects were found significant by applying a "*t*" test. The same behaviour has been previously found in other studies (Poirier et al., 1987; Vázquez et al., 1992 a and 1992 b).

The ability of the equations for reproducing the experimental results is very satisfactory: under the conditions of Table 4, the mean of the absolute value of deviations ranges from 0.4%

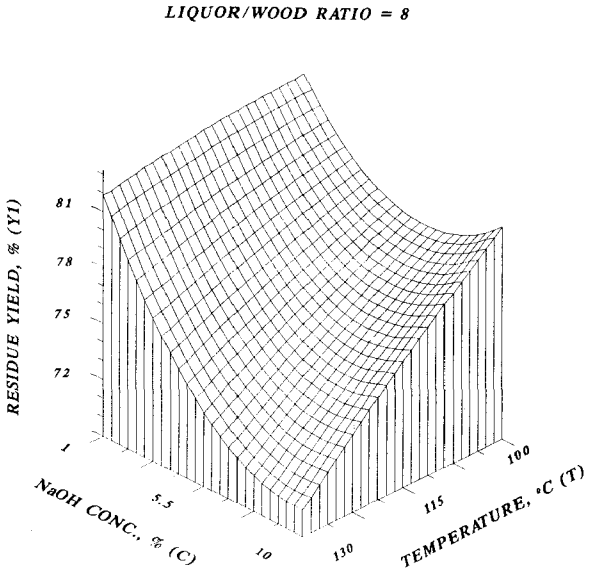

Fig. 1. Dependence of the residue yield (y_1) on temperature (T) and on NaOH concentration (C) for extractions performed using liquor/wood ratio = 8

for variable y_1 up to 3.5% for variable y_4. In order to check the ability of the models for prediction (using experimental conditions different from those used to develop the models), a new set of experiments was performed. Table 6 shows the operational conditions considered (expressed using dimensionless variables) and the experimental and calculated values obtained for the dependent variables.

Effect of the operational variables on yield and on the chemical composition of residues

In order to provide an easier understanding of the interrelationhips between dependent and independent variables, the following discussion is based on figures obtained from the empirical models using the coefficients of Table 5.

High residue yields (y_1 in the range 82.1–85.7%) were obtained in extractions using the lowest NaOH concentration (Experiments 2, 5 and 13 of Table 4, Experiment 8 of Table 6). The effect of the liquor/wood ratio was of little importance. An increase in temperature and/or alkali

Table 6. Operational conditions, experimental and calculated results obtained for the dependent variables in the experiments performed to check the reliability of the mathematical models

Independent variables				Dependent variables							
				Experimental values				Calculated values			
Exp	x_1	x_2	x_3	y_1	y_2	y_3	y_4	y_1	y_2	y_3	y_4
1	1	-1	1	73.4	67.2	49.9	48.8	70.0	67.9	50.5	51.8
2	1	0	0	70.0	65.3	51.5	33.5	72.8	64.0	49.7	34.3
3	1	1	-1	78.7	62.6	50.7	23.9	78.4	65.0	51.5	25.2
4	-1	1	1	80.0	62.3	49.3	32.6	81.1	62.0	52.8	29.8
5	-1	0	0	78.7	62.4	48.9	28.3	78.5	60.3	46.0	29.5
6	0	-1	0	74.7	63.0	49.2	33.0	77.1	58.9	47.2	36.1
7	0	0	1	77.7	62.4	49.2	45.3	75.6	64.4	49.9	42.3
8	0	0	-1	82.1	60.6	47.5	22.7	82.8	59.7	46.7	23.8

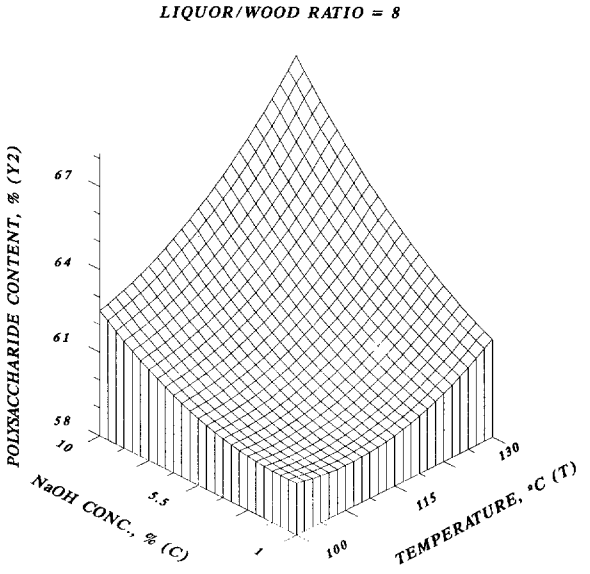

Fig. 2. Dependence of polysaccharide content of processed samples (y_2) on temperature (T) and on NaOH concentration (C) for extractions performed using liquor/wood ratio = 8

concentration resulted in decreased yields (see Figure 1). It can be noted a linear variation of yield with temperature. The effect of the NaOH concentration on y_1 was more important in the range 1–5.5% alkali. Under strong operational conditions (T = 130 °C, C = 10%), residue yields in the range 70.3–73.4% were obtained (Experiment 14 of Table 4, Experiment 1 of Table 6).

Alkaline extraction resulted in low delignification of pine wood. The lignin contents of treated samples ranged from 29.7 (under the conditions of Experiment 1 of Table 6) up to 34% (under the conditions of Experiment 5 of Table 4). In some experiments, the removal of extracts and hemicelluloses from wood resulted in solid residues with lignin contents (expressed as percent of dry weight) higher than that corresponding to untreated wood (30.2%). The limited variation range of the lignin content and the influence of the experimental error hindered a quantitative evaluation of the delignification process. The degree of delignification achieved, defined as the percent of lignin removed from solid phase after extraction in relation to the lignin present in untreated wood, ranged from 3.5% in Experiment 5 of Table 4 and 34.4% in Experiment 14 of Table 4.

Figure 2 shows the dependence of the polysaccharide content of treated wood (y_2) on temperature and on NaOH concentration using LWR = 8. The polysaccharide content increased with the vigour of the operational conditions. Experiences carried out using the highest temperature and alkali concentration (Experiment 14 of Table 4, Experiment 1 of Table 6) led to y_2 higher than 67%. Samples with reduced polysaccharide content (54.6–60.8%) were obtained when two of the studied independent variables were fixed in their lowest studied values (Experiments 1, 2 and 5 of Table 4).

The combined effects of the residue yield and the polysaccharide content of samples can be studied using a new variable, the "Percent of Polysaccharide Retained in solid phase" (PPR), defined to measure the proportion of polysaccharides remaining in the residue after extraction in relation to the amount of polysaccharides contained in the untreated wood. Such a variable can be calculated using the equation:

$$PRP = \frac{y_1 \cdot y_2}{y_{20}}$$

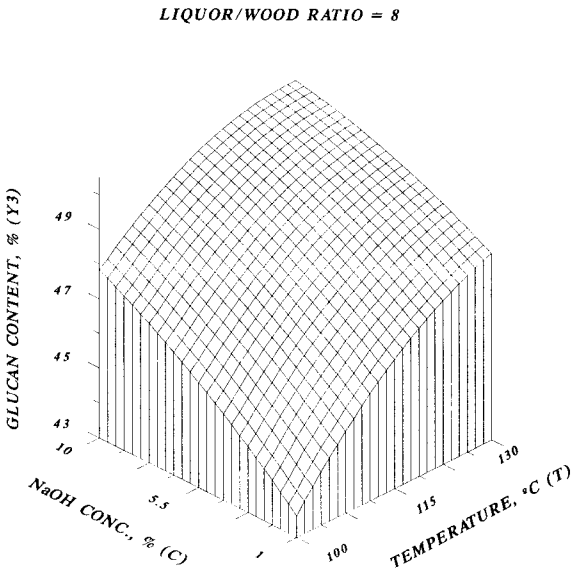

Fig. 3. Dependence of the glucan content of processed samples (y_2) on temperature (T) and on NaOH concentration (C) for extractions performed using liquor/wood ratio = 8

where y_{20} is the polysaccharide content of untreated wood (60.5%), and variables y_1 and y_2 are as above. The reduced variation range observed for PPR (77–81%) is owing to the counteraction among the variations of y_1 and y_2: stronger operational conditions resulted in decreased yields and increased polysaccharide content, with little modifications of their combined effect measured by PPR.

The glucan content of samples (y_3) was introduced as a dependent variable in order to allow a separate study of the behaviour of the cellulose and hemicellulose fractions during the extraction. In the same way previously discussed for y_2, mild experimental conditions led to samples with reduced glucan content (44.1% in Experiment 2 of Table 4). Stronger operational conditions resulted in increased glucan content of samples (see Figure 3). The increase of the glucan content with the vigour of the experimental conditions is higher than that of the polysaccharide content, owing to the preferential solubilization of the hemicelluloses. In this way, the glucan/polysaccharide ratio was 0.709 in untreated wood, 0.725 in wood treated under mild conditions (Experiment 2 of Table 4) and 0.847 in wood treated under strong conditions (Experiment 12 of Table 4). In experiences performed at 130 °C using 5.5% NaOH, samples with 51.5–52.7% glucan were obtained (Experiment 2 of Table 6, Experiment 12 of Table 4).

As it was previously dicussed for the variable PPR, a new variable ("Percent of Glucan Retained in solid phase", PGR) can be defined to give an estimation of the combinded effect of the yield and the glucan content of samples on the extraction process. Such a variable can be calculated using the equation:

$$PGR = \frac{y_1 \cdot y_3}{y_{30}}$$

where y_{30} is the glucan content of untreated wood (42.9%) and y_1 and y_3 are as above. Figure 4 shows the dependence of PGR on the NaOH concentration and on the liquor/wood ratio. A smooth minimum of PGR (86%) was observed for experiments carried out at 115 °C, in comparison with 77% of polysaccharide retention under the same experimental conditions.

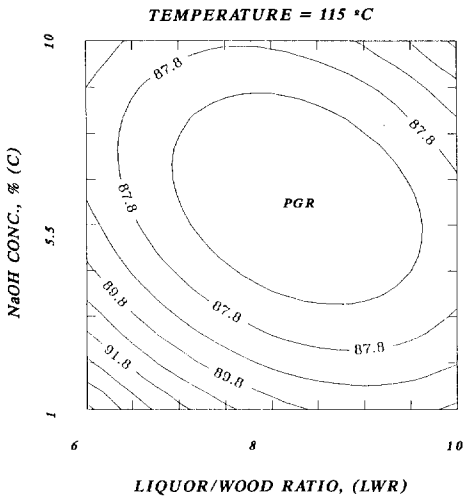

Fig. 4. Dependence of the percent of glucan retained in solid phase (PGR) on the liquor/wood ratio (LWR) and on the NaOH concentration (C) for extractions carried out at 115 °C

Study of the enzymatic hydrolysis of alkali-extracted samples

The enzymatic hydrolyses of extracted residues lasted 48 h. During the reaction, samples were taken from the reaction media at given time intervals, filtered and analyzed. The data were fitted to an hyperbolic model (Holtzapple et al., 1984). The mathematical equation proposed is:

$$X = X_{\infty} \cdot \frac{t}{t + t_{1/2}}$$

where X is the conversion achieved at time t, X_{∞} is the conversion reached at $t = \infty$ and $t_{1/2}$ is the time necessary to achieve the 50% of X_{∞}. The parameters $t_{1/2}$ and X_{∞} were calculated by nonlinear regression using the least-squares method. Figure 5 shows the experimental data and the model curve in a typical hydrolysis run. The values of X_{∞} determined in the different experiments were considered as the maximum yields, and used directly as variable y_4 in the development of the empirical models. These values resulted 0–10% higher than the experimental yields determined in samples taken at 48 h reaction time.

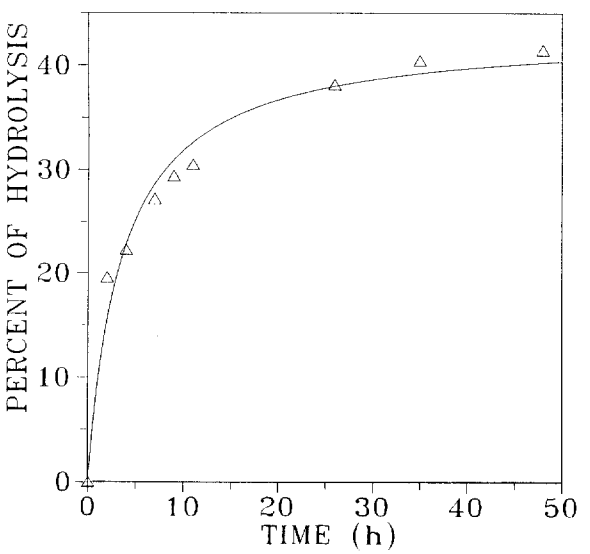

Fig. 5. Experimental and predicted dependence of the enzymatic conversion with time in a typical hydrolysis run

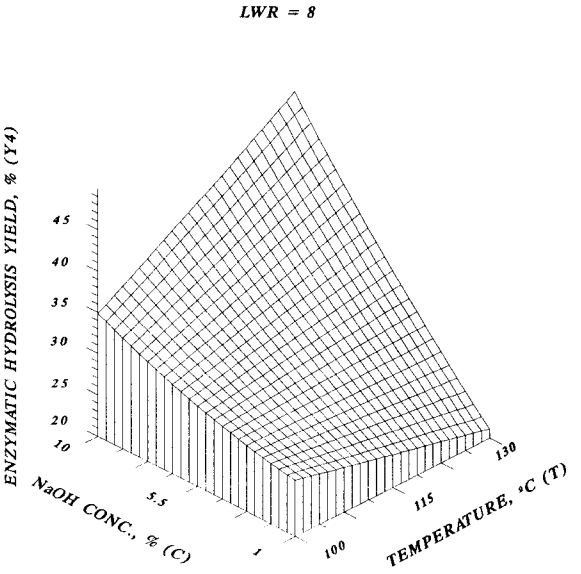

Fig. 6. Dependence of the enzymatic hydrolysis yield (y_4) on temperature (T) and on NaOH concentration (C) for extractions performed using liquor/wood ratio = 8

Low hydrolysis yields (y_4 in the vicinity of 22%) were found in Experiment 13 of Table 4 and Experiment 8 of Table 6, which were performed using the lowest NaOH concentration (1%). Figure 6 shows the dependence of the enzymatic hydrolysis yield y_4 on T and C when LWR = 8. It can be noted that the most marked increases in enzymatic hydrolysis yields were reached when increasing the NaOH concentration in experiments performed at 130 °C.

In addition to y_4, the hyperbolic model provided the regression parameter $t_{1/2}$, which was used to calculate the initial reaction rates corresponding to the different experiments of Tables 4 and 6. These initial reaction rates (r_0, expressed in mol/L.h) can be calculated from the experimental and regression data using the following equation:

$$r_0 = C_{s\,max} \cdot \left(\frac{y_2}{100}\right) \cdot \left(\frac{y_4}{100}\right) \cdot \left(\frac{1}{t_{1/2}}\right)$$

where $C_{s\,max}$ gives the sugar concentration (mol/L) obtained in the hydrolysis medium after theoretical saccharification of a substrate containing 100% polysaccharides, and the other variables are as above.

The initial reaction rates varied in the range 0.01–0.03 mol/L.h. Owing to experimental and fitting errors, the results obtained for r_0 did not show a coherent variation pattern. The highest initial rates were obtained using samples treated under mild conditions, or in experiments performed with samples obtained under conditions corresponding to the central point of the design. This behaviour is thought to be related to the loss of amorphous, easily hydrolyzable polysaccharides from the surface of solid phase when the samples were treated at high temperatures and/or with concentrated alkali solutions. High temperatures and/or alkali concentrations promoted the swelling of cellulose, leading to increases in conversion at long reaction times.

Conclusions

The ability of alkaline treatments to improve the susceptibility of *Pinus pinaster* wood to enzymatic hydrolysis was studied using empirical models derived from experimental data. The effects of three operational variables used to specify the alkaline extraction conditions (temperature, liquor/wood ratio and NaOH concentration) on residue yield, solid phase composition and enzymatic hydrolysis yield were established.

Samples with 29.7–34% lignin, 54.6–67.4% polysaccharides and 44.1–52.7% glucan at 70.0–85.7% residue yield were obtained under the operational conditions studied. The residue yield of extraction was found to be mainly dependent on temperature and NaOH concentration. Increased polysaccharide and glucan contents were obtained when the vigour of the experimental conditions was increased. Little variations were predicted for the polysaccharide fraction retained in solid phase after extraction (77–81%). The models predicted a narrow variation range in the percent of glucan remaining in treated samples (86–94% of the amount contained in untreated wood samples).

The enzymatic hydrolysis yields determined in the various experiments performed, expressed as percent of the potential sugars available, varied within the range 22.5–49.8%. Improved saccharification yields were predicted for samples pretreated at 130 °C with concentrated NaOH solutions. The initial hydrolysis rates varied from 0.01 to 0.03 mol/L.h. Stronger operational conditions did not improve the initial hydrolysis rates.

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