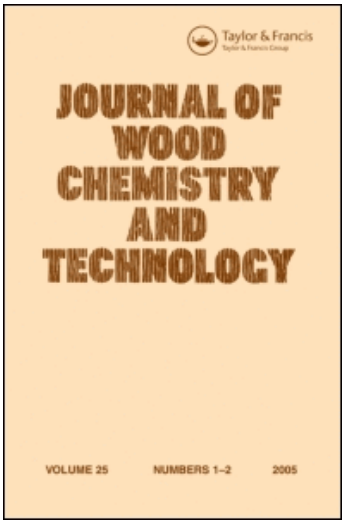

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ANALYSIS OF KINETIC DATA IN THE ENZYMATIC HYDROLYSIS OF DELIGNIFIED WOOD

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ABSTRACT

Pinus pinaster wood samples were delignified in HCl-catalyzed, acetic acid media and treated with dilute NaClO solutions to obtain a cellulosic residue highly susceptible to enzymatic hydrolysis. A set of hydrolysis experiments was carried out during 48 hours using various enzyme/substrate and liquor/solid ratios. The experimental results were fitted to empirical models, and the equations obtained were used to estimate the conversions achieved at selected reaction times (9 and 48 hours). In order to obtain a generalized interpretation of data, these calculated conversions were correlated with the operational conditions used for pretreatment and enzymatic hydrolysis. This information was used to develop a calculation procedure providing a close reproduction of the experimental data.

INTRODUCTION

The polysaccharide fraction of wood can be hydrolyzed in reaction media using acids or enzymes as catalysts. As it is well known, the enzymatic hydrolysis of wood requires a pretreatment step to improve both the kinetics and yields of saccharification. During the past few years, the authors studied the delignification of hardwoods and softwoods with HCl-catalyzed acetic acid solutions.[1-3] Under mild experimental conditions, both extensive delignification and hemicellulose degradation can be reached

in a single treatment, leaving solid residues with high cellulose content. Since the chemical composition of solid residues was favorable, acetic acid-delignified samples were assayed as substrates for enzymatic hydrolysis. However, poor results were obtained in all the experiments performed owing to the structure of the cellulosic fraction: the chemical treatments were unable to cause structural modifications (for example, reductions in crystallinity and polymerization index, or increases in available surface area and water-holding ability) necessary for improving the enzymatic hydrolysis. In order to modify these aspects, the effects of a subsequent treatment of delignified wood samples with dilute NaClO solutions were studied. It can be noted that the hypochlorite solutions provided additional delignification, although the main objective was to induce structural modifications in substrates. Because of this, the NaClO concentrations used in this work were substantially lower than those necessary to improve the enzymatic hydrolysis of acid-prehydrolyzed wood.[4]

The evaluation of processes for the enzymatic hydrolysis of pretreated wood should be carried out on the basis of both technical and economic aspects. For this purpose, the availability of generalized kinetic models should be very valuable. These kinetic models should provide a reliable interpretation of the effects caused by the operational variables (used for defining the pretreatment and/or the hydrolysis stages) on the course with time of the enzymatic saccharification.

Both theoretical and empirical models have been proposed for the mathematical interpretation of the enzymatic hydrolysis. Since this reaction was dependent in several complex phenomena, empirical models are a valid approach to the kinetic modeling. In this field, the hyperbolic model[5,6] provides an easy and useful calculation procedure.[7,8] Other authors[9,10] suggested that the hydrolysis substrates were composed by two cellulose fractions having different susceptibility to saccharification.

The purpose of this work was to develop a calculation scheme allowing a generalized interpretation of a process for wood utilization including both the pretreatment and enzymatic hydrolysis stages. Wood samples were delignified with acetic acid-water-HCl solutions under conditions selected on the basis of reported results,[3] and subsequently treated with NaClO solutions. A set of hydrolysis experiments was carried out, using the processed solid residues as substrates. In the design of experiments, the NaClO concentration used for processing the wood samples, the liquor substrate ratio employed in saccharification, the enzyme/substrate ratio and the reaction time were considered as

experimental variables. The [sugar concentration]/time series of data obtained in individual experiments were fitted to a kinetic model based on the following assumptions: i) two cellulosic fractions existed in wood, showing a different susceptibility toward the enzymatic hydrolysis, and ii) both fractions reacted with the same hyperbolic pattern. The equations derived from these hypotheses were used to predict the polysaccharide conversion achieved after selected reaction times (9 and 48 hours) in individual experiments. The calculated conversions were correlated with the operational conditions according to a generalized calculation procedure that provided a close reproduction of experimental data.

MATERIALS AND METHODS

Raw material

Pinus pinaster wood chips were obtained from a local particleboard mill, milled to pass a 0.5 mm screen and air-dried. The wood meal was homogenized in a single lot to avoid differences among aliquots and stored in polyethylene bags.

Composition of wood

Wood samples were subjected to quantitative saccharification with 72% H_2SO_4 following standard methods.[11] The solid residue was considered as lignin. The liquors from quantitative saccharification were neutralized and analyzed for glucose and sugars as reported elsewhere.[12]

Chemical processing of wood

Wood samples were treated at 130 °C during 0.5 hours in media containing 95 weight percent of acetic acid and 0.45 weight percent of HCl, using a liquor/solid ratio of 8 g/g. The solid residues were washed with acetone, air-dried and subjected to treatments with dilute NaClO solutions. The hypochlorite concentration used in treatments (HC) was considered as an operational variable, and fixed in 0.06, 0.34 or 0.62 mol

NaClO/L solution depending on the experiment considered. The reaction time (2 hours), temperature (40 °C) and the liquor/solid ratio (10 g/g) were kept constant in all the experiments. The initial pH of media was regulated to 8.[4] Processed samples were subjected to quantitative saccharification (using the same method than that employed for raw wood). The composition of solids was characterized by their contents in lignin, total polysaccharides and glucan. The glucan content provided a reliable estimate of the cellulose contained in substrates. The hemicellulose content of samples can be estimated from the difference between their percentages in polysaccharides and glucan.

Enzymatic hydrolysis

Delignified and NaClO-treated samples were used as substrates for hydrolysis experiments, which were carried out in stirred Erlenmeyer flasks at 48.5 °C during 48 hours. Mixtures of commercial cellulases (from *Trichoderma reesei*) and β-glucosidase (from *Aspergillus niger*) were used as catalysts. Enzyme concentrates ("Celluclast 1,5 L" and "Novozym 188") were a kind gift from Novo España (Barcelona, Spain). The [β-glucosidase activity]/[cellulase activity] ratio was fixed in 13 International Units/Filter Paper Unit, and both the enzyme/substrate ratio (ESR) and the liquor/solid ratio (LSR) used in the hydrolysis step were considered as operational variables. For the variable ESR, the values selected were 3, 8 or 13 Filter Paper Unit/g substrate, whereas for LSR the values slected were 12, 20 or 28 g liquor/g substrate. At selected reaction times (0, 2, 4, 6, 9, 16, 20, 24, 28, 36, 42 and 48 hours), samples were withdrawn from the reaction media, filtered and analyzed for reducing sugars by the Somogyi-Nelson method.[11]

Correlation of data

The [sugar concentration]/time series of data were fitted to the proposed kinetic models by nonlinear regression using commercial software (TableCurve from Jandel Scientific, Corta Madera, CA). The generalization of the kinetic parameters was done with statistical software (SPSS).

RESULTS AND DISCUSSION

Wood composition and chemical processing of the raw material

Table 1 lists the results obtained on the composition of raw wood, delignified residues and NaClO-processed samples. The lignin contents were measured as the insoluble residues after quantitative saccharification of substrates, whereas the total polysaccharide content and the glucan content were calculated from the concentrations of total sugars and glucose determined in the liquors produced in the analytical assays. Table 1 also includes results on the residue yield of treatments. From the data listed in this Table, it can be calculated that the treatments in acetic acid media removed 91% of the initial lignin and 81% of initial hemicelluloses, whereas the recovery of cellulose (measured as glucan) accounted for the 80% of the amount contained in untreated wood. As it has been stated above, the main purpose of the hypochlorite treatments was to cause structural alterations in substrates rather than to provoke changes in chemical composition. The oxidizing treatments caused both hemicellulose and lignin degradation, being the delignification very significant in experiments performed with 0.34 or 0.62 mol NaClO/L. However, it can be noted that the least hypochlorite concentration assayed (0.06 mol/L) was enough to markedly improve to susceptibility of samples to hydrolysis. A quantitative evaluation of the influence of the NaClO concentration on the hydrolysis stage is included below. It merits to be remarked the high selectivity of the process proposed toward cellulose degradation: Only 19-22% of the initial glucan (which provides an estimate of cellulose) was lost during the two steps (delignification-swelling) of chemical treatment, a very good result considering the extensive removal of lignin and hemicelluloses.

Enzymatic hydrolysis

Wood samples delignified in acetic acid media and swelled with NaClO solutions were used as substrates for enzymatic hydrolysis. As it is said above, the effect of the NaClO concentration used in the oxidizing treatment (HC) and both the enzyme/substrate ratio (ESR) and the liquor/solid ratio (LSR) used in hydrolyses were considered as experimental variables.

TABLE I

Data on wood composition and wood processing*

a) Wood composition (as g/100 g of oven-dried wood)

Lignin, 30.2
Polysaccharides, 60.5
Glucan, 42.9

b) Residue yield and composition of delignified samples:

Residue yield: 42.3 g/100 g untreated wood, o. d. basis.
Composition of solid residue (as g/100 g delignified wood):
Lignin, 6.3
Polysaccharides, 89.6
Glucan, 81.5

c) Residue yield of hypochlorite treatments

NaClO conc (mol/L)	Yield (g/100 g delignified wood)
0.06	85.0
0.34	84.7
0.62	84.3

d) Composition of delignified and NaClO-treated samples

NaClO conc. (mol/L)	Lignin**	Polysaccharides**	Glucan**
0.06	5.8	97.2	93.3
0.34	1.8	98.5	96.7
0.62	1.4	99.1	97.5

* Contents in glucan and polysaccharides expressed as glucose and reducing sugar equivalent, from analysis of hydrolyzates.
**Compositions expressed as g/100 g of delignified and NaClO-treated samples, o. d. basis)

Table 2 lists the operational conditions corresponding to the set of experiments performed. It can be noted that 23 hydrolysis trials explored 21 different combinations of the independent variables, with two additional replicates in the central point of the experimental design to measure the experimental error. This protocol was developed on the basis of a second order, incomplete, factorial design of experiments,[13] that was completed with selected additional assays to improve the reliability results.

The [sugar concentration]/time series of data obtained in a given experiment after t hours of reaction (SC(t)) were used to estimate the corresponding polysaccharide conversion (X(t)) using the equation:

$$X(t) = 100 . \frac{SC(t)}{PSC} \qquad (eq.\ 1)$$

where PSC denotes the "potential sugar concentration", defined as the concentration corresponding to the quantitative conversion of the polysaccharide fraction of substrate into sugars. The values of PSC can be calculated for each experiment from material balances, taking into account both the composition of processed wood samples (listed in Table 1) and the liquor/solid ratio (LSR) employed in the considered experiment.

Kinetic modeling of individual hydrolysis assays

The hyperbolic model[5,6] correlated the conversion X(t) with the reaction time by the expression:

$$X(t) = \frac{t . X(\infty)}{t+\tau} \qquad (eq.2)$$

where $X(\infty)$ is the conversion predicted for an infinite reaction time, and τ is the reaction time necessary for achieving 50% of $X(\infty)$. This model provided a good interpretation of our results at prolonged reaction times, but systematic errors were found in the predictions corresponding to the earlier stages of reaction, a fact hindering the generalization of results. A modified model was assayed, based on the assumption that the cellulose contained in substrates was composed by two fractions: an "easy" fraction (corresponding to the α percent of cellulose) and a "difficult" fraction (amounting for the (100-α) percent of cellulose). Assuming that both fractions reacted with hyperbolic

TABLE 2

Operational Conditions Assayed, Expressed in Terms of the Variables ESR, LSR and HC Defined in Text; Kinetic Parameters τ^e and τ^d Obtained by Regression of Experimental Results; Statistical Parameters Measuring the Correlation and Significance of Equations and Values Calculated for Variables X(9) and X(48) from the Kinetic Model

	OPERATIONAL CONDITIONS			REGRESSION PARAMETERS		STATISTICAL PARAMETERS		CALCULATED VARIABLES	
Exp.	ESR, FPU/g	LSR, g/g	HC mol/L	τ^e,h	τ^d, h	R^2	F_{exp}*	X(9)	X(48)
1	3	12	0.34	7.32	966	0.9992	12154	11.8	21.1
2	3	20	0.06	4.82	338	0.9957	2353	15.1	28.1
3	3	20	0.62	5.14	143	0.9987	7875	17.5	38.2
4	3	28	0.34	7.19	169	0.9971	3447	15.2	35.1
5	8	12	0.06	1.86	260	0.9995	18262	19.3	31.7
6	8	12	0.62	2.10	253	0.9984	6248	19.0	31.9
7	8	20	0.34	2.10	115	0.9988	8568	22.0	42.7
8	8	20	0.34	2.97	121	0.9966	2963	20.6	41.5
9	8	20	0.34	2.57	123	0.9985	6613	21.0	41.4
10	8	28	0.06	5.66	39.0	0.9966	2930	27.3	62.0
11	8	28	0.62	4.28	30.3	0.9975	3931	31.9	67.4
12	13	12	0.34	0.87	101	0.9992	12955	24.8	45.4
13	13	20	0.06	1.06	46.6	0.9906	1058	30.8	60.2
14	13	20	0.62	1.35	25.7	0.9987	7746	38.1	71.5
15	13	28	0.34	2.10	27.8	0.9994	1601	35.8	69.9
16	8	28	0.34	5.20	37.3	0.9954	2211	28.2	63.1
17	3	20	0.34	10.2	250	0.9982	5434	12.2	29.4
18	13	20	0.34	1.63	42.8	0.9973	3741	30.8	61.6
19	8	20	0.06	1.02	119	0.9918	1202	23.6	42.6
20	13	12	0.62	0.71	67.2	0.9973	3655	28.0	53.0
21	3	28	0.62	4.71	116	0.9918	1192	18.9	41.6
22	3	12	0.06	6.26	1060	0.9848	649	12.5	21.2
23	13	12	0.06	0.92	81.7	0.9992	12232	26.1	49.2

*F_{exp} defined as the ratio between the mean squares of model and error.

kinetics, the evolution with time of the polysaccharide conversion was expressed by the equation:

$$X(t) = \frac{\alpha . t}{t+\tau^e} + \frac{(100-\alpha) . t}{t+\tau^d} \qquad (eq.\ 3)$$

where X(t), α and t are as above, and τ^e and τ^d are the reaction times necessary for achieving the 50% conversion of the "easy" and "difficult" fractions of cellulose, respectively.

A preliminary analysis of results was conducted taking α, τ^e and τ^d as regression parameters. Wide variation ranges were observed for both τ^e and τ^d, but it was observed that α reached similar values in all the experiments. So, α was fixed in its mean value (α = 20), and new regression calculations were made taking τ^e and τ^d as optimization parameters, according to the expression:

$$X(t) = \frac{20 . t}{t+\tau^e} + \frac{80 . t}{t+\tau^d} \qquad (eq.\ 4)$$

Table 2 lists the set of kinetic parameters calculated, as well as the corresponding statistical coefficients R^2 and F_{exp}, measuring the correlation and significance of models, respectively. Additionally, Figure 1 shows the close agreement existing between the experimental and calculated conversions.

In order to generalize the kinetic model (eq. 4), a systematic study on the dependence of both τ^e and τ^d on the operational conditions was performed. Empirical, second-order models were assayed to describe the effects of the independent variables on τ^e and τ^d, using the same procedure reported elsewhere.[8] With this calculation scheme, statistically significant equations giving a good interpretation of the most part of experiments were derived. However, the wide variation ranges of the dependent variables resulted in negative kinetic parameters for two experiments. This fact made the correlations unable for quantitative predictions of some experiments.

A detailed analysis of the deviations between experimental and predicted results suggested that the lack of accuracy observed in the calculation scheme described above was caused by compensation effects of the "easy" and "difficult" fractions of substrates at intermediate reaction times, and that a better correlation of data could be obtained if predicted conversions were used instead τ^e and τ^d for correlating the effect of the operational conditions.

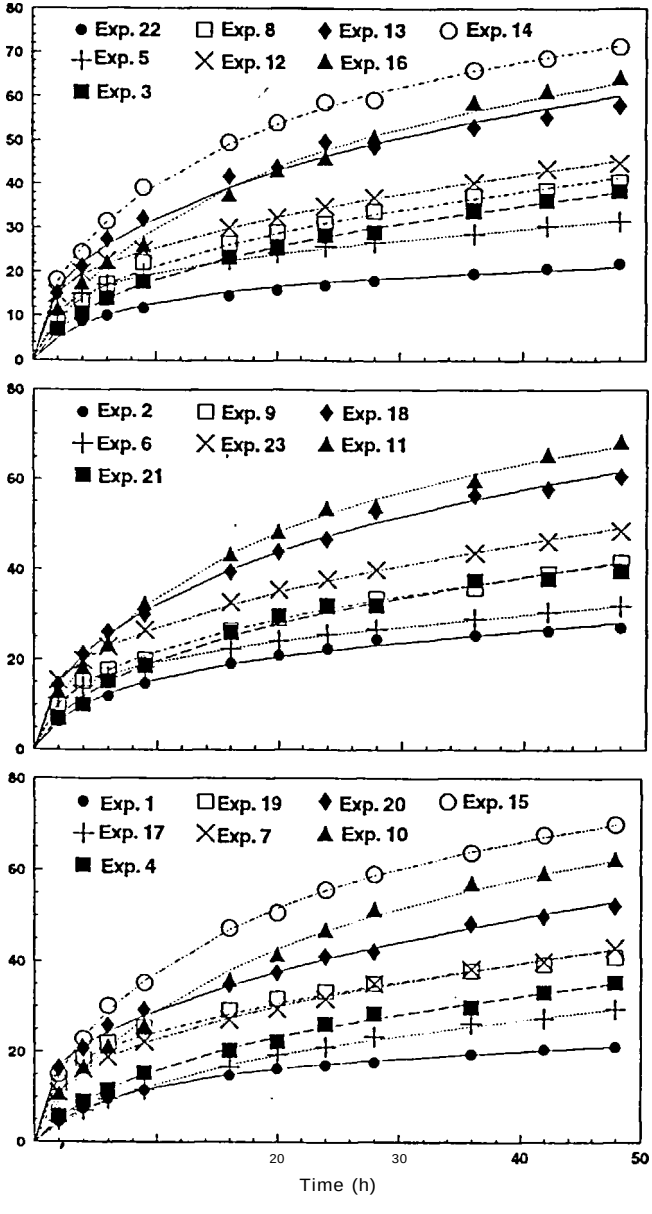

FIGURE 1. Experimental and calculated polysaccharide conversions corresponding to the experiments performed.

On this basis, the predicted polysaccharide conversions for the various experiments after 9 and 48 hours of reaction time (X(9) and X(48), respectively) were calculated for experiments 1 to 23 taking into account the kinetic parameters of Table 2, using the corresponding expressions derived from eq. 4:

$$X(9) = \frac{20.9}{9+\tau^{e}} + \frac{80.9}{9+\tau^{d}} \qquad (eq.\ 5)$$

$$X(48) = \frac{20.48}{48+\tau^{e}} + \frac{80.48}{48+\tau^{d}} \qquad (eq.\ 6)$$

The results obtained for these variables are listed in Table 2. For calculation purposes, both X(9) and X(48) were considered as a function of three normalized, dimensionless, dependent variables defined as follows:

$$N^{ESR} = \frac{ESR-8}{5} \qquad (eq.7)$$

$$N^{LSR} = \frac{LSR-20}{8} \qquad (eq.8)$$

$$N^{HC} = \frac{HC-0.34}{0.28} \qquad (eq.9)$$

where N^{ESR}, N^{LSR} and N^{HC} are the normalized enzyme substrate ratio, normalized liquor/solid ratio and normalized hypochlorite concentration, respectively. The values of these normalized, independent variables can be easily calculated for each experiment from the corresponding values of ESR, LSR and HC listed in Table 2.

Empirical models were used to correlate the dependent variables X(9) and X(48) with the dimensionless, independent variables defined in eqs. 5 to 7, according to the expression:

$$X_j = b_{0j} + b_{1j}.N^{ESR} + b_{2j}.N^{LSR} + b_{3j}.N^{HC} +$$
$$b_{12j}.N^{ESR}.N^{LSR} + b_{13j}.N^{ESR}.N^{HC} + b_{23j}.N^{LSR}.N^{HC} +$$
$$b_{11j}.(N^{ESR})^2 + b_{22j}.(N^{LSR})^2 + b_{33j}.(N^{HC})^2 \qquad (eq.10)$$

TABLE 3

Regression Coefficients, Statistical Parameters and Significance of Coefficients Obtained by Correlation of Variables X(9) and X(48) with the Dimensionless, Normalized Independent Variables.

a) Regression coefficients

	VARIABLE	
COEFFICIENT	X_1 or X(9)	X_2 or X(48)
b_0	21.8	43.2
b_1	9.1	16.4
b_2	4.7	13.0
b_3	1.5	2.9
b_{12}	2.0	2.8
b_{13}	1.0	0.8
b_{23}	1.0	0.8
b_{11}	0.1	0.7
b_{22}	-0.2	0.3
b_{33}	2.9	4.7

b) Statistical parameters measuring the significance and correlation of the empirical models.

	VARIABLE	
PARAMETER	X_1 or X(9)	X_2 or X(48)
F_{exp}*	79	31
Prob $[F_{exp} > F_{et}]$*	<0.01	<0.01
R^2	0.9821	0.9552
Adj. R^2	0.9697	0.9241

c) Significance of coefficients:

c.1) Variable X(9):

Significant coefficients at the 95% confidence level: b_{11}, b_{21}, b_{31}, b_{121}, b_{111}

Significant coefficients at the 90% confidence level: b_{131}, b_{231}

c.2) Variable X(48):

Significant coefficients at the 95% confidence level: b_{12}, b_{22}, b_{32}, b_{122}

Significant coefficients at the 90% confidence level: b_{122}

*F_{exp} as in Table 2. F_{et} defined as the statistical value of F for the degrees of freedom of model and error.

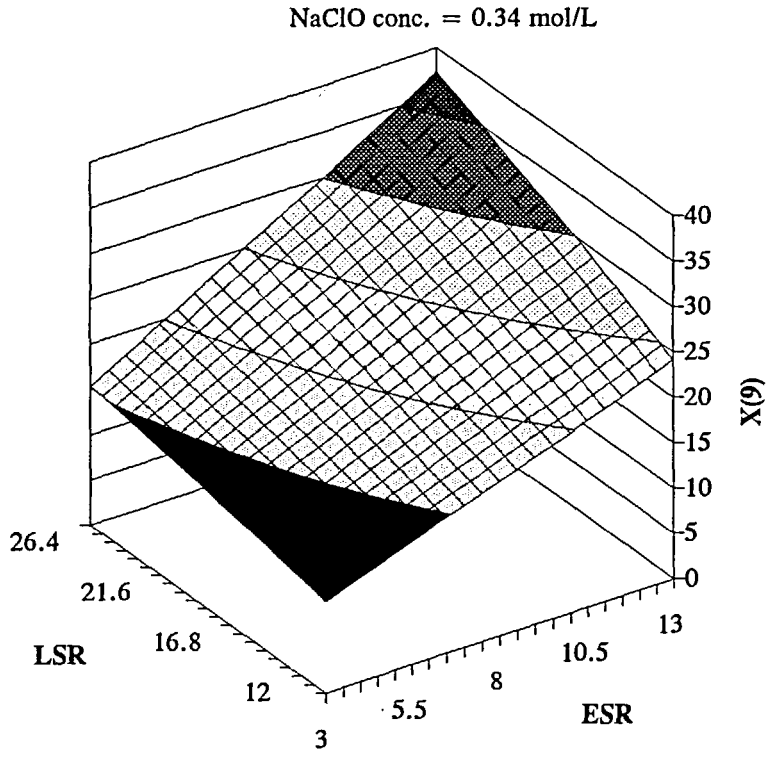

FIGURE 2. Calculated dependence of the polysaccharide conversion achieved after 9 hours of reaction time [X(9)] on the ezyme/substrate ratio ESR and on the liquor/solid ratio LSR for samples treated with 0.34 mol hypochlorite/L.

where X_j denotes the dependent variable considered [X_1=X(9); X_2= X(48)], and the terms $b_{0j}...b_{33j}$ are regression coefficients calculated from the experimental data by the least-squares method.

Table 3 lists the set of regression coefficients obtained for X(9) and X(48), as well as additional information on the significance of models (*F* test), correlation (R^2) and significance of the several coefficients within the considered model (*t* test). It can be noted the excellent degree of significance and the high correlation found for the dependence of both X(9) and X(48) on the operational conditions.

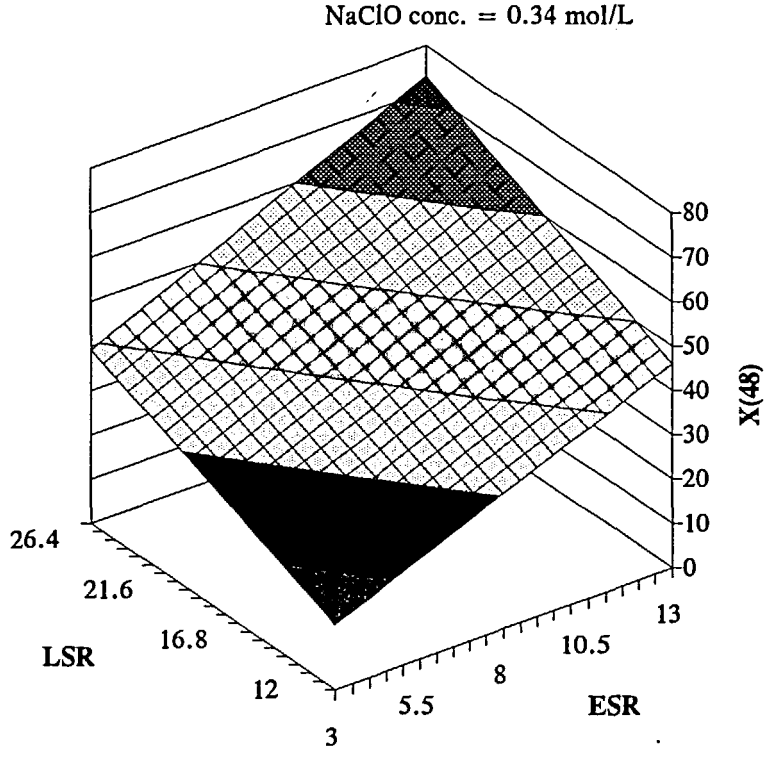

FIGURE 3. Calculated dependence of the polysaccharide conversion achieved after 48 hours of reaction time [X(48)] on the ezyme/substrate ratio ESR and on the liquor/solid ratio LSR for samples treated with 0.34 mol hypochlorite/L.

Since the independent variables used in correlation were normalized, the absolute values of the coefficients of Table 3 give a measure of the influence of the considered term on the measured effect. The variable X(9) was mainly influenced by ESR and LSR, and the major contributions corresponded to the linear terms of the model. Figure 2 shows the calculated dependence of X(9) on LSR and ESR when the hypochlorite concentration HC was fixed in its mean value. The variable X(48) was mainly influenced by ESR, whereas the effects caused by HC were higher than those found for X(9). As in the previous case, the linear terms of the model gave the main contributions to the dependent variable. Figure 3 shows the calculated dependence of X(48) on LSR and ESR

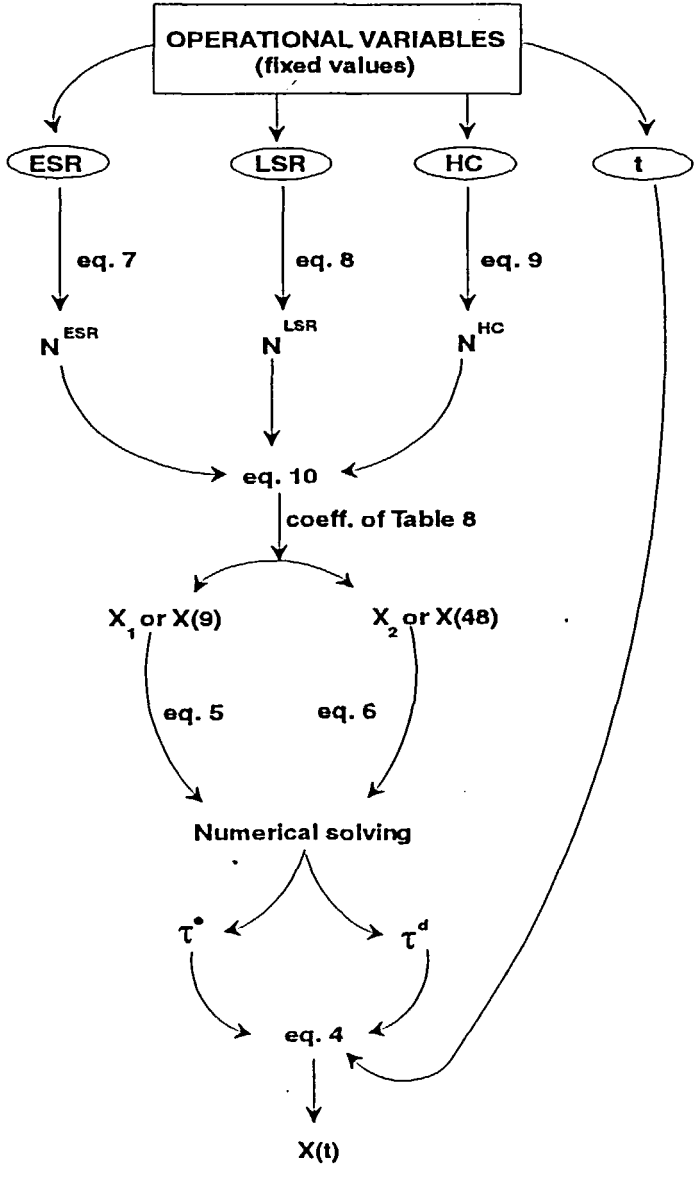

FIGURE 4. Calculation scheme proposed for the calculation of the polysaccharide conversion achieved under fixed operational conditions.

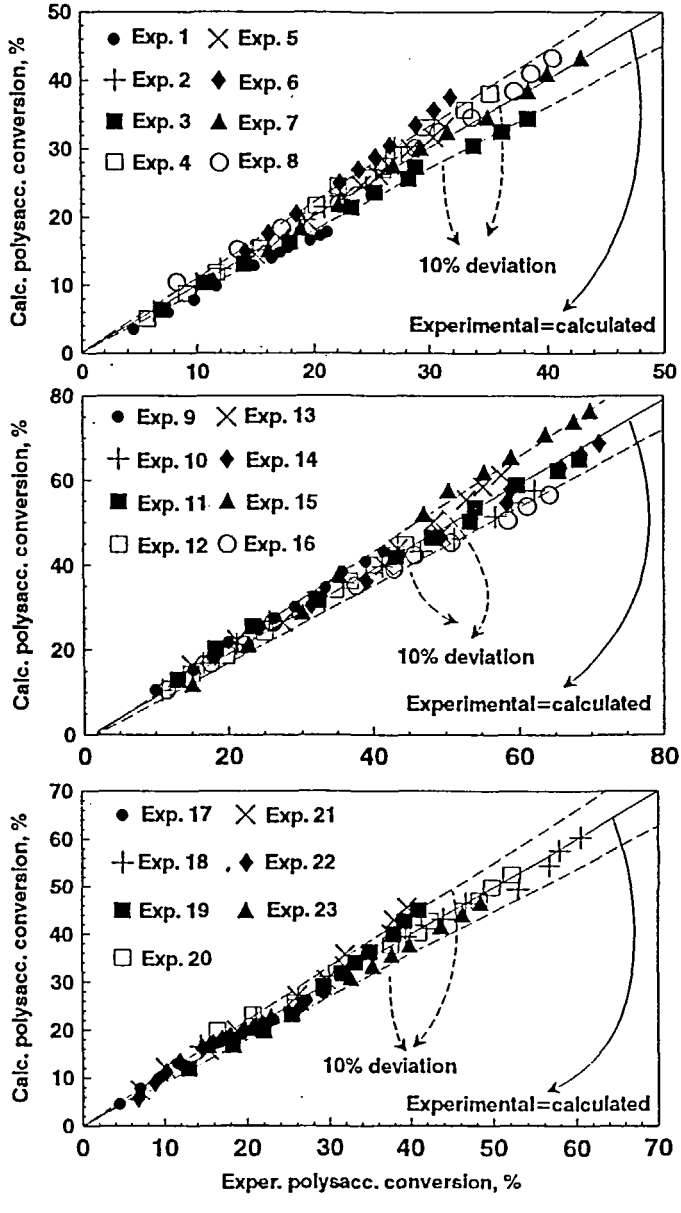

FIGURE 5. Agreement between experimental and calculated polysaccharide conversions corresponding to the experiments performed.

for experiments performed with 0.34 mol NaClO/L. Figures 2 and 3 show the almost linear dependence existing between the calculated conversions and the most influential operational variables.

The information obtained was used to provide a generalized interpretation of data, according to the calculation scheme shown in Figure 4. For given values of the experimental variables ESR, LSR and HC (which have to be selected within their respective variation ranges), the correspondent normalized, dimensionless variables N^{ESR}, N^{LSR} and N^{HC} can be calculated using their respective definitions. From these results, and considering the set of regression coefficients of Table 3, the empirical models allow the estimate of X(9) and X(48). If the calculated conversions are substituted in eq. 5 and 6, a system of two equations is obtained, where τ^c and τ^d are the only unknown variables. These equations can be solved to provide the values of both τ^c and τ^d, which can be then substituted in eq. 4 to obtain the conversion predicted for a given treatment time.

In order to assess the validity of this approach, the above calculation scheme was utilized to reproduce the data corresponding to the operational conditions assayed (see Figure 5): the reduced deviations existing between experimental and calculated results in all the experiments confirmed the ability of the calculation framework proposed for quantitative predictions. So, the analysis of data carried out in our work, based on a reasonable amount of experimental deal, provided information useful to perform a comparative evaluation of the experimental conditions on technical or economic basis.

CONCLUSIONS

Sequential treatments of pine wood with acetic acid-water-HCl mixtures and dilute NaClO solutions resulted in solid residues with high glucan content and good susceptibility toward the enzymatic hydrolysis. A laboratory protocol was developed to evaluate the dependence of selected operational conditions on the kinetics of the enzymatic hydrolysis, using three selected experimental variables. The results of individual experiments were fitted to a modified hyperbolic model, which served to predict the conversions achieved at given reaction times. These conversions were correlated with the operational conditions, leading to a generalized calculation scheme that provided close reproduction of the experimental data. The procedure proposed for the

analysis of data gives a useful assessing for the simulation and evaluation of the studied process.

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