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Production of a cellulosic substrate susceptible to enzymatic hydrolysis from prehydrolyzed barley husks

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An effective process for the chemical-biotechnological utilization of barley husks is reported. A first treatment with sulfuric acid (prehydrolysis) allowed the solubilization of hemicelluloses to give xylose-containing liquors (suitable to make fermentation media for xylitol production) and a solid phase containing cellulose and lignin. The solid residues from prehydrolysis were treated with NaOH in order to increase their cellulase digestibility. In the alkaline treatments, the effects of temperature (in the range, 50–130ºC), reaction time (10–60 min) and NaOH concentration (3–10 weight percent of solution) on the composition of solid residues were assessed by means of an experimental plan with factorial structure. The cellulose content increased with temperature and NaOH concentration, whereas the duration of treatments was not influential within the range tested. The treated samples showed high susceptibility toward the enzymatic hydrolysis with cellulases, leading to almost quantitative glucose yields under selected operational conditions.

Key words: barley husks, hydrolysis, alkali treatment, cellulose, xylitol

Introduction

Barley husk is an agricultural byproduct whose direct utilization as a carbohydrate source (for example, as feed supplement or for manufacture of glucose or ethanol) is hindered by its low digestibility. On the other hand, the combustion of barley husk is difficult owing to its comparatively high ash content. In this scope, the sequential processing of barley husk with sulfuric acid and NaOH allows the separation of two fractions (hemicellulose as soluble sugars in the first processing step and a cellulosic solid phase in the second processing step) which can be utilized by fermentation and enzymatic hydrolysis, respectively.

The processing of lignocellulosic substrates in acidic media (prehydrolysis) is carried out to convert the hemicellulose polysaccharides (xylan, mannan and galactan) into the correspondent monosaccharides (xylose, mannose and glucose). As xylan is the main hemicellulosic polymer in barley husk, xylose is the major sugar present in the prehydrolysis liquors obtained from this raw material. After neutralization and

Manuscript received May 2001

nutrient supplementation, the hydrolyzates can be used as fermentation media to produce xylitol, a low caloric pentitol with sweetening power which has negative heat of solution, anticariogenic properties and is suitable as sugar substitute for diabetics (Parajó et al. 1997, Cruz et al. 2000a). Using continuous fermentation with cell recycle, the production of xylitol from barley husk hydrolyzates can be carried out with productivities as high as 2.53 g/L · h (Cruz et al. 2000b).

Owing to the selective hemicellulose removal caused by prehydrolysis, the solid residue from this processing step is enriched in both cellulose and lignin. In order to convert it in a suitable substrate for enzymatic hydrolysis, a delignification stage (for example, with NaOH) must be performed. As lignin forms a physical barrier hindering the access of enzymes to cellulose, delignification should result in increased accessibility, and so in an improved glucose yield with faster reaction rate. Alkaline treatments achieve other collateral effects (for example, alteration in the physicochemical features of cellulose such as crystallinity and surface area) leading to improved susceptibility toward enzymatic hydrolysis (Gharpuray et al. 1983).

This study deals with the chemical-biotechnological processing of barley husk. In a first step, the hemicellulosic fraction was converted into xylose-containing liquors suitable for xylitol production. The solid phase from prehydrolysis was treated with NaOH under a variety of operational conditions, and the effects of treatments on both composition and hydrolysis susceptibility of cellulosic substrates were measured.

Material and methods

Raw material

Barley husk was kindly provided by San Martín (Ourense, Spain), where husks were separated from the grain by pneumatic conveying and cyclone separation. Husks were stored in a dry and dark place at room temperature until utilization.

Analysis of the raw material

Aliquots from the homogenized lots were subjected to moisture determination and to quantitative hydrolysis in two-stage, acid treatment (the first step with 72 weight percent sulfuric acid at 30ºC during 1 hour, and the second one after dilution of the media to 4 weight percent sulfuric acid at 121ºC during 1 hour) (Vázquez et al. 1991). The solid residue after hydrolysis was considered to be Klason lignin. Hydrolyzates were assayed by HPLC using an Interaction ION-300 column (mobile phase, H_2SO_4 0.01 M; flow rate, 0.4 mL/min; IR and UV detection). This method allowed the direct determination of glucose, xylose, arabinose, acetic acid, ethanol, xylitol, furfural and hydroxymethylfurfural (HMF). Representative material balances are presented in Figure 1.

Chemical processing of samples

Ground samples of barley husks were hydrolyzed under selected conditions (3% H_2SO_4, 15 min, 130°C, liquid:solid ratio of 8:1 g/g). The solids from treatments were separated by filtration, washed with water, air dried and treated in autoclave with solutions containing 3–10% NaOH at 50–130ºC during 10–60 min. In this step, the liquor/solid ratio was fixed in 10 g/g. At the end of treatments, the solid residues were separated by filtration, washed with water, air dried and analyzed as described for the raw material.

Enzymatic hydrolysis

The commercial enzyme concentrates ("Celluclast" and "Novozym", with cellulase and β-glucosidase acitivities, respectively) used in experiments were kindly provided by Novo, Denmark.

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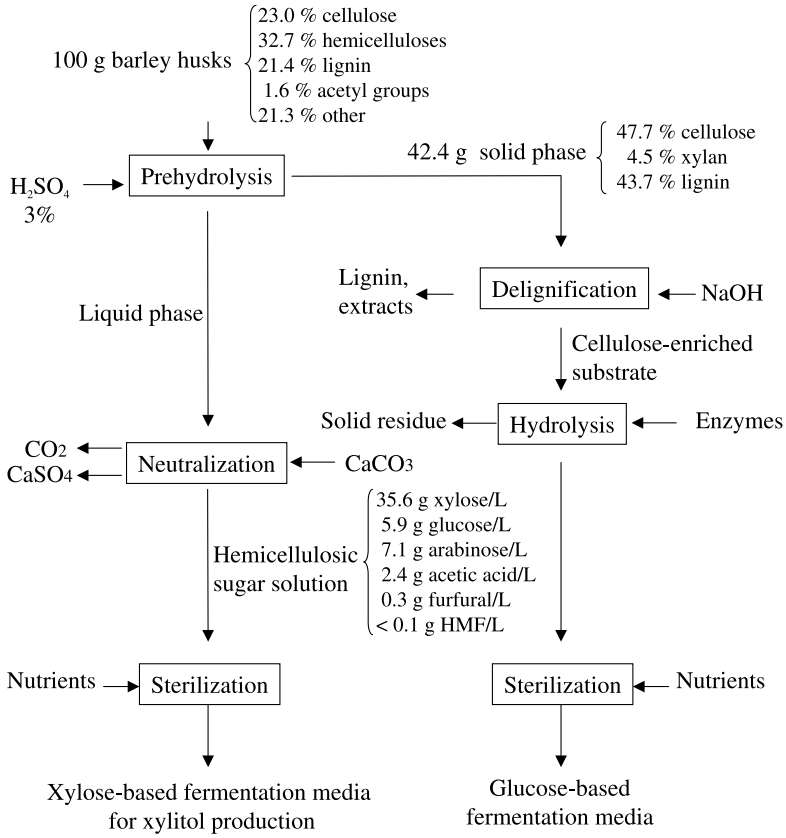

Fig. 1. Scheme proposed for the chemical-biotechnological processing of barley husks.

The cellulase activity of concentrates was assayed by the Filter Paper Activity test (FPA) according to Mandels et al. (1976) and expressed as Filter Paper Units (FPU)/mL. The β-glucosidase activity was measured according to Paquot and Thonart (1982). The operational conditions used in enzymatic hydrolysis were: temperature, 48.5ºC; pH, 4.85; liquor/solid ratio, 30 g/g; cellulase/substrate ratio, 28 FPU/g and cellobiase/cellulase ratio, 13 IU/FPU (Moldes et al. 2000).

Experimental design and statistical analysis

In the alkaline stage of barley husk processing, an incomplete 3^3 factorial design (Box et al. 1978) was used to study the influence of temperature, reaction time and NaOH concentration on both the composition of the solid substrates from treatments and their susceptibility to enzymatic hydrolysis. The experimental data were analyzed by the Response Surface methodology using the Statistica 5.0 software. The interrelationship between dependent and operational variables was established by a model including linear, interaction and quadratic terms:

$$Y = b_0 + b_1 \cdot x_1 + b_2 \cdot x_2 + b_3 \cdot x_3 + b_{12} \cdot x_1 \cdot x_2 + b_{13} \cdot x_1 \cdot x_3 + b_{23} \cdot x_2 \cdot x_3 + b_{11} \cdot x_1^2 + b_{22} \cdot x_2^2 + b_{33} \cdot x_3^2$$

where Y is the dependent variable, b denotes the regression coefficients (calculated from experimental data by multiple regression using the least-squares method), and x denotes the independent variables.

Results and discussion

The sequential treatment of barley husk with sulfuric acid and sodium hydroxide allows the benefit of hemicelluloses and cellulose. Figure 1 shows some details on the composition of liquors and solid residues from treatments. The hydrolyzates coming from the first step have been successfully employed for the production of xylitol, and the corresponding results have been reported in a previous study (Cruz et al. 2000b). Using cell recycle after membrane separation, the fermentation of hydrolyzates with the yeast *Debaryomyces hansenii* led to a maximum volumetric productivity of 2.53 g/L · h at a dilution rate of 0.284 h^{-1}.

In order to assess the possibility of reaching a simultaneous benefit of both liquid and solid phases coming from the prehydrolysis step, preliminary enzymatic hydrolysis assays were carried out using the solid residues from prehydrolysis as substrates. As expected, these substrates showed a poor susceptibility toward enzymatic hydrolysis (data not shown) owing to their high lignin content. In order to improve the results, a delignification stage with NaOH was carried out before the enzymatic hydrolysis.

The independent variables used in this study and their variation ranges were: temperature T, 50–130ºC; duration of treatments t, 10–60 min and NaOH concentration [NaOH], 3–10 weight percent of solution. The standardized (coded) adimensional variables employed, having variations limits (–1,1), were defined as x_1 (coded temperature), x_2 (coded time) and x_3 (coded NaOH concentration). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits (see Table 1).

Table 1 also lists the dependent variables considered: the composition of delignified solids was measured by variables y_1 (cellulose content of samples, g/100 g oven-dry substrate), y_2 (hemicellulose content of samples, g/100 g oven-dry substrate) and y_3 (Klason lignin content of samples, g/100 g oven-dry substrate); whereas y_4 (defined as conversion of cellulose into glucose) was selected to measure the susceptibility of prehydrolyzed, alkali-treated samples toward enzymatic hydrolysis. It can be noted that the processed samples also contained other fractions different from those measured by y_1, y_2 and y_3 (such as acid-soluble lignin, acetyl groups, ashes, etc.) which were of minor importance for this study.

Table 1. Variables used in this study.

Variable	Nomenclature	Units	Variation range
Independent variables			
Temperature	T	ºC	50–130
Time	t	Min	10–60
NaOH concentration	[NaOH]	wt %	3–10
Dimensionless, coded independent variables			
Dimensionless temperature	x_1	(T – 90)/40	(–1,1)
Dimensionless time	x_2	(t – 35)/25	(–1,1)
Dimensionless NaOH concentration	x_3	([NaOH] – 6.5)/3.5	(–1,1)
Dependent variables			
Cellulose content, g /100 g o. d. sample	y_1		
Hemicellulose content, g /100 g o. d. sample	y_2		
Lignin content, g /100 g o. d. sample	y_3		
Cellulose conversion into glucose, g glucose/100 g potential glucose	y_4		

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Table 2. Operational conditions considered in this study (expressed in terms of the coded independent variables dimensionless temperature x_1, dimensionless time x_2 and dimensionless NaOH concentration x_3) and experimental results achieved for the dependent variables y_1 (cellulose content of samples, %), y_2 (hemicellulose content of samples, %), y_3 (lignin content of samples, %) and y_4 (cellulose conversion into glucose, %).

	Operational conditions			Experimental results			
Exper.	x_1	x_2	x_3	y_1	y_2	y_3	y_4
1	0	–1	–1	68.3	5.2	24.0	89.6
2	0	1	–1	64.2	3.0	27.0	99.7
3	0	–1	1	71.0	2.8	22.5	91.9
4	0	1	1	79.8	2.5	16.2	91.0
5	–1	–1	0	68.2	4.6	24.0	79.6
6	–1	1	0	69.3	4.2	23.0	86.5
7	1	–1	0	80.1	3.0	15.6	96.0
8	1	1	0	86.1	4.2	6.8	91.9
9	–1	0	–1	64.5	6.3	26.0	77.6
10	–1	0	1	70.2	3.4	23.5	86.8
11	1	0	–1	75.9	3.0	18.8	93.6
12	1	0	1	84.7	3.0	11.4	94.3
13	0	0	0	75.0	3.1	19.0	99.7
14	0	0	0	75.0	3.1	17.5	98.8
15	0	0	0	74.8	2.9	18.6	100.4

Since a systematic study of the effects caused by the operational variables on composition and hydrolysis susceptibility would require a great amount of experimental work, an incomplete, factorial design of experiments was carried out. Several research groups used phenomenological models based on experimental designs to study the chemical processing and/or bioconversion of lignocellulosic materials (Parajó et al. 1995, Roberto et al. 1995, Alves et al. 1998, Mayerhoff et al. 1998, Silva and Roberto 1999). In this study, we utilized an incomplete factorial design, in which 3 dependent variables were assayed at 3 levels. Based on experimental data, equations including linear, interaction and quadratic terms were employed to describe the interrelationship between operational and experimental variables.

Table 2 shows the set of experimental conditions assayed (expressed in terms of coded variables), as well as the experimental data obtained for variables y_1 to y_4. The sequence for the experimental work was randomly established to limit the influence of systematic errors on the interpretation of results. It can be noted that experiments 13–15 are replications in the central point of the design measuring the experimental error.

Table 3 lists the regression coefficients and their statistical significance (based on a t-test). The same Table includes statistical parameters (r^2 and F) measuring the correlation and the statistical significance of the models, respectively. It can be noted that all the models showed good statistical parameters for correlation and significance and allowed a close reproduction of experimental data.

In the range tested, the reaction time caused only minor effects on the cellulose content of samples, as it can be seen from the absolute value of the corresponding coefficients. Figure 2 shows the predicted dependence of the cellulose content of samples (y_1) on the most influential operational variables (T and [NaOH]) in experiments lasting 35 min. Increased severity (defined by high values of temperature and/or NaOH concentration) resulted in remarkable increases in the cellulose content of samples. The effects caused by increased alkali concentrations were

Table 3. Regression coefficients, significance level and statistical parameters (r^2 and F) measuring the correlation an significance of the models.

a) Regression coefficients and significance

Coefficients	y_1	y_2	y_3	y_4
b_0	74.93*	3.033*	18.37*	99.63*
b_1	6.84*	–0.663*	–5.49*	5.66*
b_{11}	2.00*	0.758*	–1.76*	–8.06*
b_2	1.46*	–0.213*	–1.64*	1.50*
b_{22}	–1.03*	0.208**	0.74	–3.08*
b_3	4.10*	–0.725*	–2.78*	0.42
b_{33}	–3.11*	0.133	3.32*	–3.53*
b_{12}	1.22*	0.400*	–1.95*	–2.76*
b_{13}	0.78*	0.725*	–1.23**	–2.14*
b_{23}	3.23*	0.475*	–2.33*	–2.76*

* Significant coefficients at the 95 % confidence level
** Significant coefficients at the 90 % confidence level

b) Statistical parameters measuring the correlation and significance of models

Variable	R^2	Corrected R^2	F_{exp}	Significance level (based on the F test)
y_1	0.9942	0.9839	90.99	>99%
y_2	0.8779	0.6582	47.81	>99%
y_3	0.9753	0.9310	5.25	>95%
y_4	0.9414	0.8359	20.29	>99%

more marked when the experiments were carried out at the highest temperature assayed. Under the severest conditions considered (130ºC, 10% NaOH), the model predicted cellulose contents as high as 86%, confirming the suitability of the alkaline processing for delignification.

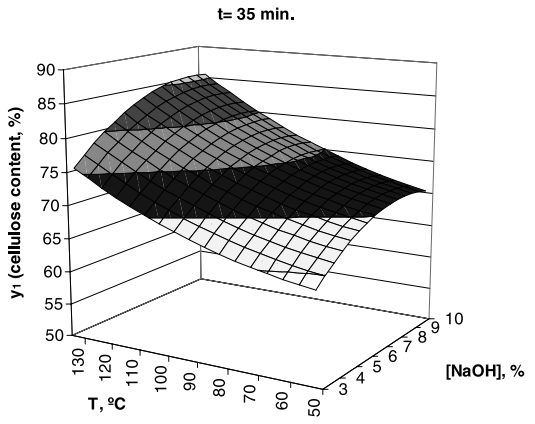

Fig. 2. Dependence of the cellulose content of samples (variable y_1) on NaOH concentration and temperature predicted for samples delignified for 35 min.

The experimental results achieved for the hemicellulose content of samples (measured by y_2) varied within a narrow range (2.9–6.3%). The surface response shown in Figure 3 (calculated for assays lasting 35 min) shows a continuous decrease in y_2 with temperature for experiments with 3% NaOH. However, the model predicted a slight increase in the hemicellulose content of samples with temperature, which can be justified on the basis of both experimental and fitting errors. Operating at 130ºC, the hemicellulose content of samples remained almost constant with the NaOH concentration, confirming that the hemicellulose solubilization was completed at high temperature even in media containing the lowest NaOH concentration tested.

Figure 4 shows the predicted dependence of variable y_3 (lignin content of samples) on the most influential operational variables (T and [NaOH]) for treatments lasting 35 min. As ex-

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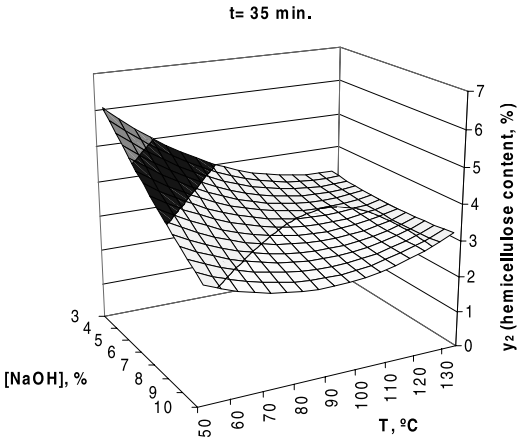

Fig. 3. Dependence of the hemicellulose content of samples (variable y_2) on NaOH concentration and temperature predicted for samples delignified for 35 min.

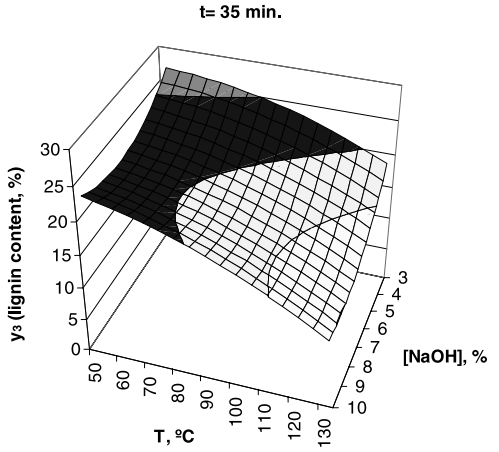

Fig. 4. Dependence of the lignin content of samples (variable y_3) on NaOH concentration and temperature predicted for samples delignified for 35 min.

pected, the response surface predicted lower lignin contents for higher temperatures at a given NaOH concentration. Comparatively, the effects caused by the NaOH concentration on the lignin content were of minor importance.

The most important variable for the objectives of this work was the cellulose conversion achieved in the enzymatic hydrolysis step (y_4). The experimental data listed in Table 2 show that all the alkali-treated samples were highly susceptible toward the enzymatic hydrolysis, with 77.6–100% cellulose conversion into glucose. Cellulose conversions below 86% were obtained only in experiments 5 and 9, which corresponded to assays performed at the lowest temperature considered (50ºC) with either short treatments (x_2 = –1 in experiment 5) or low NaOH concentrations (x_3 = –1 in experiment 9). When low-temperature delignification was combined with either prolonged reaction times (such as in experiment 6) or high NaOH concentrations (such in experiment 10), the cellulose conversion increased significantly (y_4 up to 86.5–86.8%). Harsher delignification conditions resulted in increased susceptibility toward enzymatic hydrolysis. The experimental results of Table 2 show that enzymatic hydrolysis yields higher than 93% can be obtained under a variety

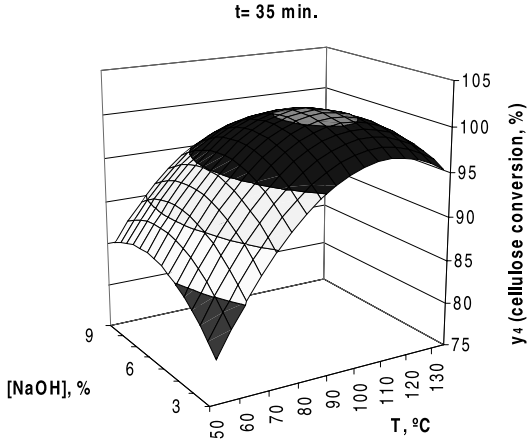

Fig. 5. Dependence of the cellulose conversion into glucose (variable y_4) on NaOH concentration and temperature predicted for samples delignified for 35 min.

of operational conditions. Figure 5, which shows the predicted dependence of the cellulose conversion on the operational variables T and [NaOH], confirms the above findings: the major effects on y_4 are caused by temperature, particularly in the range 50–100ºC. The decreased cellulose conversions predicted at high temperatures and alkali concentrations can be justified on the basis of both experimental and fitting errors. For treatments lasting 35 min, 94–100%

cellulose conversion is predicted for samples delignified at temperatures above 80ºC even in treatments carried out with the minimum NaOH charge considered.

In conclusion, the sequential treatment of barley husk with sulfuric acid and sodium hydroxide allows a simultaneous benefit of the hemicelluloses and cellulose fractions to produce xylitol and glucose solutions respectively. The prehydrolyzed barley husk, after being subjected to an alkaline treatment, shows high cellulose content (up to 86%) and shows an excellent susceptibility toward enzymatic hydrolysis, with near quantitative glucose yields.

Acknowledgements. Authors are grateful to "Xunta de Galicia" (Project XUGA 38303A98) and to the University of Vigo (Project 64502 K904) for the financial support of this work, as well as to Ms. Aida Ramos Nespereira and Antonia Rodríguez Jardón for their excellent technical assistance.

References

Alves, L.A., Felipe, M.G.A., Silva, J.B.A.E., Silva, S.S. & Prata, A.M.R. 1998. Pretreatment of sugarcane bagasse hemicellulose hydrolysate for xylitol production by *Candida guilliermondii*. *Applied Biochemistry and Biotechnology* 70–72: 89–98.

Box, G.E.P., Hunter, W.G. & Hunter, J.S. 1978. *Statistic for experimenters: an introduction to design, data analysis and model building*. John Wiley, New York. p. 125–175.

Cruz, J.M., Domínguez, J.M., Domínguez, H. & Parajó, J.C. 2000a. Preparation of fermentation media from agricultural wastes and their bioconversion into xylitol. *Food Biotechnology* 14, 1&2: 79–97.

Cruz, J.M., Domínguez, J.M., Domínguez, H. & Parajó, J.C. 2000b. Xylitol production from barley bran hydrolysates by continuous fermentation with *Debaryomyces hansenii*. *Biotechnology Letters* 22: 1895–1898.

Gharpuray, M.M., Fan, L.T. & Lee, Y.H. 1983. Caustic pretreatment study for enzymatic hydrolysis of wheat straw. *Wood and Agricultural Residues* 1: 369–389.

Mandels, M., Andreotti, R. & Roche, C. 1976. Measurement of saccharifying cellulose. *Biotechnology Bioengineering Symposium* 6: 21–33.

Mayerhoff, Z.D.V.L., Roberto, I.C. & Silva, S.S. 1998. Production of xylitol by *Candida mogii* from rice straw hydrolysate. Study of environmental effects using statistical design. *Applied Biochemistry and Biotechnology* 70–72: 149–159.

Moldes, A.B., Alonso, J.L. & Parajó, J.C. 2000. Multi-step feeding systems for lactic acid production by simultaneous saccharification and fermentation of processed wood. *Bioprocess Engineering* 22: 175–180.

Paquot, M. & Thonart, Ph. 1982. Hydrolyse enzymatique de la cellulose regenerée. *Holzforschung* 36: 177–181.

Parajó, J.C., Alonso, J.L. & Santos, V. 1995. Enzymatic hydrolysis of wood: an engineering assessment. *Bioprocess Engineering* 12: 253–261.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. 1997. Improved xylitol production with *Debaryomyces hansenii* Y-7426 from raw or detoxified wood hydrolyzates. *Enzyme Microbiology Technology* 21: 18–24.

Roberto, I.C., Sato, S., Mancilha, I.M. & Taqueda, M.E.S. 1995. Influence of media composition on xylitol fermentation by *Candida guilliermondii* using response surface methodology. *Biotechnology Letters* 17, 11: 1223–1228.

Silva, C.J.S.M. & Roberto, I.C. 1999. Statistical screening method for selection of important variables on xylitol biosynthesis from rice straw hydrolysate by *Candida guilliermondii* FTI 20037. *Biotechnology Techniques* 13: 743–747.

Vázquez, D., Lage, M.A., Parajó, J.C. & Vázquez, G. 1991. Transformación de materiales lignocelulósicos: Composición, fraccionamiento y aprovechamiento. *Revista Agroquimica Tecnología de Alimentos* 31, 2: 143–164.