0273-2289/1559–0291 (Online)/07/136–140/141–154/$30.00

Enzymatic Hydrolysis Optimization to Ethanol Production by Simultaneous Saccharification and Fermentation

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Abstract

There is tremendous interest in using agro-industrial wastes, such as *cellulignin*, as starting materials for the production of fuels and chemicals. *Cellulignin* are the solids, which result from the acid hydrolysis of the sugarcane bagasse. The objective of this work was to optimize the enzymatic hydrolysis of the cellulose fraction of *cellulignin*, and to study its fermentation to ethanol using *Saccharomyces cerevisiae*. Cellulose conversion was optimized using response surface methods with pH, enzyme loading, solid percentage, and temperature as factor variables. The optimum conditions that maximized the conversion of cellulose to glucose, calculated from the initial dried weight of pretreated *cellulignin*, (43°C, 2%, and 24.4 FPU/g of pretreated *cellulignin*) such as the glucose concentration (47°C, 10%, and 25.6 FPU/g of pretreated *cellulignin*) were found. The *desirability* function was used to find conditions that optimize both, conversion to glucose and glucose concentration (47°C, 10%, and 25.9 FPU/g of pretreated *cellulignin*). The resulting enzymatic hydrolyzate was fermented yielding a final ethanol concentration of 30.0 g/L, in only 10 h, and reaching a volumetric productivity of 3.0 g/L·h, which is close to the values obtained in the conventional ethanol fermentation of sugar cane juice (5.0–8.0 g/L·h) in Brazil.

Index Entries: Cellulignin; enzymatic hydrolysis; ethanol production; sugarcane bagasse; cellulases; simultaneous saccharification; *saccharomyces cerevisiae*.

Introduction

Sugarcane bagasse represents the main lignocellulosic material to be considered in many tropical countries, because it is readily available in the distilleries without additional cost and has high carbohydrate and low

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lignin content *(1)*. In Brazil, sugarcane (*Saccharum* sp.) is one of the most important agro-industrial product. According to data of São Paulo State Research Foundation *(2)*, about 60–90% of the generated bagasse from milled sugar cane in the country is used as fuel for steam and energy production and, between 10 and 40% is not used, representing about 5–12 million t annually. In 2003, 340 million t of sugarcane were produced and, consequently, 91.8 millions t of bagasse were generated. Because of the high carbohydrate content, sugar cane bagasse can be potentially used for bioethanol production and/or others products, within the context of biorefinery *(3)*.

Lignocellulosic materials typically contain 55–75% of dry weight of carbohydrates, which are polymers containing sugar units of five and six carbon atoms. Sugarcane bagasse is made up of 38.1 wt% cellulose, 28.4 wt% hemicellulose, 18.4 wt% lignin, and 15.1 wt% proteins and ashes *(4)*. Cellulose is a biopolymer of β-1,4-linked glucose dimers (cellobiose). This abundant biopolymer is made up of crystalline and amorphous regions. The amorphous component is digested more easily by enzymes than the crystalline component *(5,6)*. Crystalline cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen of 1,4-β-D-glucan chains that are tightly linked by numerous hydrogen bonds, both side-to-side and top-to-bottom in a lattice like manner *(6)*.

Hemicellulose are largely made up of aldopentoses (arabinose, xylose, galactose, and manose) and present crosslinking glycans, which are a sort of polysaccharides that can be linked to cellulose microfibrils by hydrogen bonds *(6,7)*. They may coat microfibrils but are also long enough to span the distance between microfibrils and link them together to form a network. Lignin is a complex macromolecule of phenolic polymer, made up of phenylpropanoid units (hydroxycinnamyl, *p*-coumaryl, coniferyl, and sinapyl alcohols that constitute most of the lignin network) *(8)*. Lignin is the most abundant noncarbohydrate constituent of lignocellulosic material. Its presence represents a major problem for the biomass conversion process because the physical structure of native lignocellulose is intrinsically resistant to enzyme attack, especially cellulose, which is further protected by the surrounding matrix of lignin, hemicellulose, and pectin *(9,10)*.

The lignocellulosic biomass must be pretreated to make the cellulose fraction more accessible to enzymatic attack. Diverse pretreatment processes have been evaluated technically and economically aiming at improving enzymatic hydrolysis, these include acid or alkali treatment, steam-explosion, and organic solvents *(11,12)*. Pretreatments are also necessary in order to use sugarcane bagasse in bioconversions, for producing several compounds such as organic acids, xylitol, and mainly ethanol, which represents a promising alternative fuel to reduce environmental problems *(13)*.

In the bioconversion of solid cellulose to a readily fermentable stream of glucose monomers, an enzymatic complex should be used. The enzymatic complex that is able to hydrolyze cellulose to glucose molecules is

called cellulases. This enzymatic complex is usually made up of three types of enzymes that act synergistically. The first is called endoglucanases (EC 3.2.1.4), which cut randomly at amorphous sites in the cellulose yielding smaller chains of cellulose called cello-dextrins *(14)*. The second enzyme group, called exoglucanases (glucohidrolase enzyme commission [EC] 3.2.1.74 and celobiohidrolase EC 3.2.1.91), degrades cello-dextrins and crystalline cellulose, thus liberating glucose and cellobiose as major product *(14,15)*. Finally, the cellobiose is hydrolyzed into glucose by β-glucosidases (EC 3.2.1.21). Currently, the costs of pretreatment and enzymes for cellulose hydrolysis are still the main economic obstacle to the commercialization of biomass bioconversion technologies *(14,15)*.

The aim of this work was to study the different factors that play an important role in the enzymatic hydrolysis of cellulose; those variables were pH, solid content, temperature, and enzymatic loading. In this context, it was required to establish the conditions to obtain optimal values for the response variables (final concentration of glucose and conversion of pretreated *cellulignin* to glucose). Finally, the evaluation of the resulting hydrolyzate fermentability was also the subject of investigation. The fermentation experiments were carried out under the optimal conditions found in the enzymatic hydrolysis stage.

Materials and Methods

Raw Material and Pretreatment of Cellulignin

The sugarcane bagasse, *Saccharum* sp., was provided by "Usina Costa Pinto," Piracicaba in São Paulo, Brazil. The *cellulignin* was obtained by acid hydrolysis of sugarcane bagasse, from which the hemicellulosic fraction was removed *(16)*. This resulting solid residue was pretreated for increasing the accessability of enzymes to cellulose, by removing partially the lignin. The conditions for this pretreatment were as follows: the *cellulignin* was mixed with a solution of NaOH 4% (w/v), which was further submitted to thermal treatment at 121°C for 30 min *(17)*. The pretreated *cellulignin* was then washed until pH 5.5 and finally, it was dried at 50°C for 24 h, this solid was called *celluligninG*.

Enzyme Activities

Filter paper activity was determined as recommended by Ghose (1987) *(18)* and it is expressed as *Filter Paper Units* (FPU) per milliliter of mixture. The activity of enzyme used, GC 220 of *Genencor International Inc.* (Leiden, The Netherlands), was 104.27 FPU/mL of mixture.

Enzymatic Hydrolysis

Pretreated *cellulignin* was hydrolyzed by using GC 220 (*Genencor International, Inc.*). The enzymatic hydrolysis was carried out in 125-mL

Table 1
Full Factorial Design (3^4)

Factor	Low label (–1)	Center label (0)	High label (+1)
1. Temperature (°C)	30	40	50
2. Enzyme loading (FPU/g *CelluligninG*)	5.0	17.5	30.0
3. pH	5.0	5.5	6.0
4. Solid (%)	2	6	10

flasks on a shaker at 150 rpm. Temperature, pH (citrate buffer), solid percent, and enzyme loading were selected as the most important variables for the optimization of the process.

Experimental Design for the Optimization of the Enzymatic Hydrolysis

Full factorial design of four factors at three levels was developed *(19)*. High, intermediate, and low levels of the factors were considered. The experimental design matrix is shown in Table 1. The analysis of the results of this design includes the computation of the linear (L), quadratic (Q), and interaction effects, and the analyses of the variances ascribed to them. The statistical significance of these effects was evaluated by using *t*-tests and *F*-tests *(19)*.

Final glucose concentration and conversion of *celluligninG* to glucose were considered as response variables for the process analysis. A quadratic model was obtained relating each response variable to the significant effects. These models were used to define the conditions that separately and simultaneously maximize the response variables. The method developed by Derringer and Suich *(20)* was adopted in the case of multiple response optimization. Their procedure makes use of the so-called *desirability* functions *(19–21)*. The STATISTICA 6.0 (Statsoft, Inc., Tulsa, OK) software was used here in order to implement all these statistical analysis.

Yeast Cultivation

A pure culture of *Saccharomyces cerevisiae* was isolated from commercial yeast *Fleischmann* in the Laboratory of Bioprocess Development at the Federal University of Rio de Janeiro, Brazil, and it was used in the fermentation assays. Inoculum was obtained in 500-mL shaker flask with a working volume of 250 mL, and a medium consisting of (g/L): glucose, 30; urea, 1.25; KH_2PO_4, 1.1; yeast extract, 1.5; salts, and citric acid solution 40 mL/L *(22)*. Glucose was sterilized separately from the others components to prevent damage to the nutritional qualities of the medium. The sterilization condition, in both cases, was 111°C for 15 min. The pH and temperature were maintained at 5.5 and 37°C, respectively, during 10 h. After cell quantification, the volume required to achieve the initial cell concentration in the bioreactor, was centrifuged at 5000 rpm for 15 min.

Fermentation Assay

Experiments were carried out in a batch bioreactor (BIOFLO III, New Brunswick Scientific, New Brunswick, NJ; 1.5 L) with 1.0 L working volume, at 37°C, 300 rpm, pH 5.0, and an initial cell concentration of 4.0 g (dry weight)/L. The hydrolyzed products obtained after the enzymatic hydrolysis were used as fermentation medium without any nutritional supplements. Three fermentations were run, each one with the hydrolyzate obtained under the conditions established by the separate and simultaneous maximization of the response variables (conversion to glucose and glucose concentration).

Analytical Methods

The cells were measured for absorbance at 570 nm and a calibration curve obtained by the dry weight method was used *(23)*. Glucose, cellobiose, and ethanol concentration were determined by high-performance liquid chromatography-Waters by using a Shodex SC1011 ion exchange column for sugars (300×8 mm^2; Shoko Co., Ltd., Tokyo) at 80°C as stationary phase and degassed Milli-Q (Molsheim, France) water as the mobile phase at a flow rate of 0.6 mL/min *(24)*.

Results and Discussion

Several delignification processes were evaluated in a previous study in order to improve or facilitate the accessibility of the enzymes to the cellulose matrix *(25)*. Of the evaluated pretreatments, the one coded as *G*, as described previously, resulted in the best performance of the enzyme preparation. This pretreatment improves 2.4 times the performance of the enzymatic hydrolysis when compared with the analog process with non-pretreated cellulignin (Fig. 1).

The results of 87 experiments, obtained by utilizing a three-level full factorial design with four factors (3^4) plus six replicates in the center value, were analyzed by considering glucose concentration and conversion to glucose as output (response) variables. The results of this analysis are shown by using the Pareto charts as they present, very clearly, the most significant effects *(21)*. In these charts, the effects represented by rectangles, which lay to the right side of the 0.05 *p*-value vertical line, are statistically significant and must be considered in the mathematical model. This *p*-value implies in a 95% level of significance, which is the usual level assumed in statistical analysis *(19,21)*.

In the Pareto chart for conversion to glucose (Fig. 2), the largest effect is owing to the enzyme loading followed by temperature, and the quadratic factors (*Q*) for these two factors, all of them affecting positively the glucose formation. In general, the interaction factors exert less significant effects over the conversion to glucose. The negative influence of the solid percentage on the conversion to glucose can be explained by the enzymatic inhibition caused by the increase on the final hydrolysis product concentration (glucose) *(14)*.

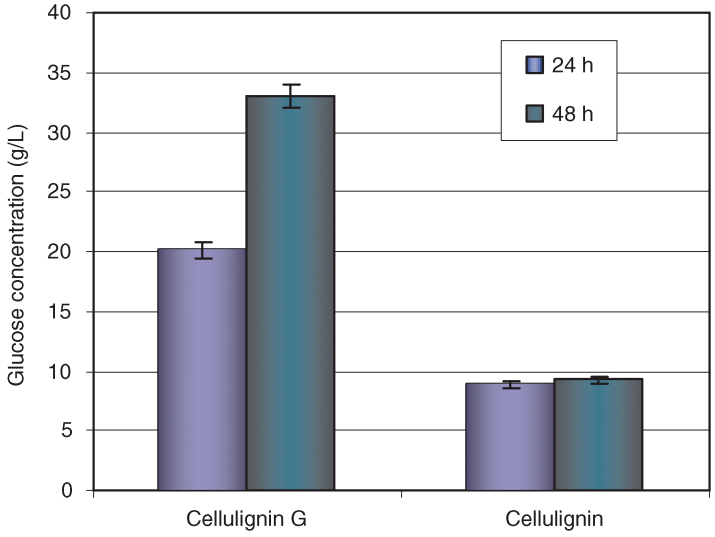

Fig. 1. Effect of pretreatment on enzymatic hydrolysis of cellulignin (*T*, 50°C; solid [%], 7; and enzyme concentration, 20 FPU/g cellulignin).

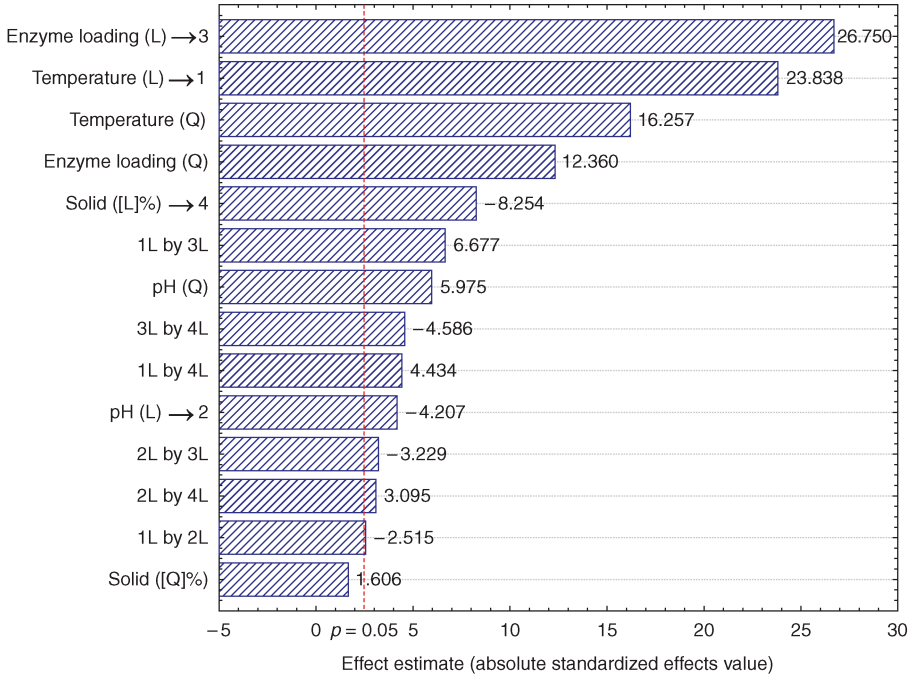

Fig. 2. Pareto chart of standardized effects; on conversion to glucose (g glucose/g *cellulignin*G).

When the Pareto chart is analyzed, concerning the glucose concentration variable (Fig. 3), the degree of importance among the factors changes. In this case, the factor that presented the largest effect was the solid percentage (Solid [%]). Similarly to the other analyzed response variable

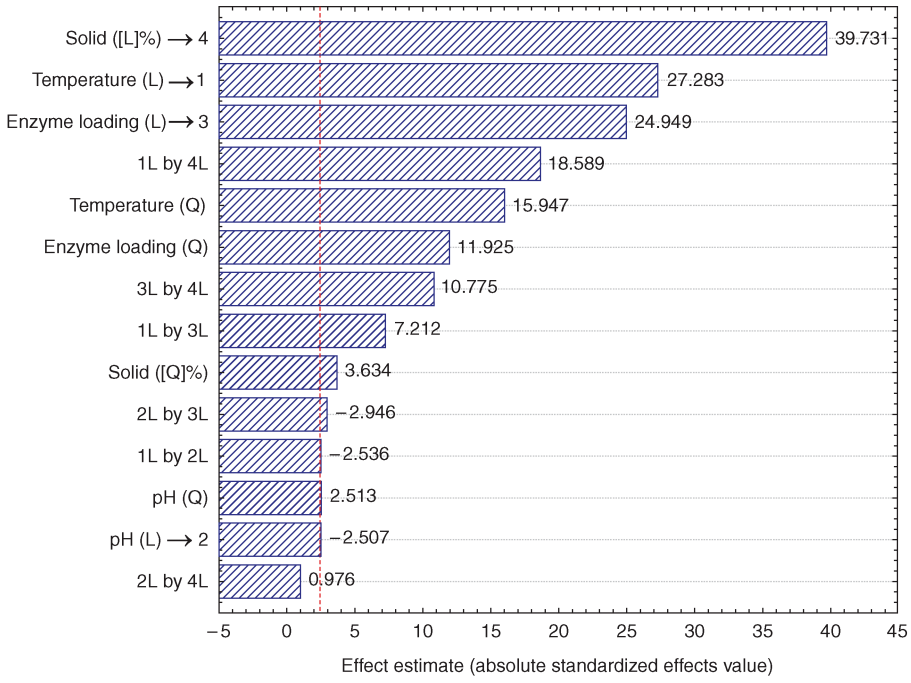

Fig. 3. Pareto chart of standardized effects; on glucose concentration (g glucose/L).

(conversion to glucose), the factors temperature and enzyme loading presented highly significant positive effects. However, unlike what was observed in the analysis of conversion to glucose, in which the interaction factors did not show much significance, the positive influence on glucose concentration of the interaction between solid percentage and temperature was evidenced here. For both output variables, it can be depicted from the Pareto chart that the pH presented a linear effect with magnitude lower than the other factors discussed previously. However, it is important to consider that pH variations, outside the range evaluated experimentally, can generate changes in the behavior of the enzyme, leading to a halt of the process.

The pH was eliminated from the model used for the optimization of the enzymatic hydrolysis, because of the fact that it affects the response variables less than the other factors in the range considered. Therefore, a value of pH = 5.0 was selected for further experiments. The use of this value is in accordance with the following facts: the majority of the work presented in literature with cellulosic enzyme preparations uses pH = 5.0 for cellulose hydrolysis; it is a pH value in which the contamination of the fermentation by bacteria is uncommon *(26)*; the yeast (*S. cerevisiae*) displays a good performance in a slightly acid pH range; and finally pH = 5.0 is within the stability range of commercial cellulases *(1,7,11,14,17,27)*.

Analyses of the variances was also performed. The analysis of significance (using *F*-tests) provided exactly the same significant factors already shown in the Pareto charts. Additionally, the percentage of variance

Table 2
Models Coefficients

	Variables	
Coefficients	Conversion to glucose	Glucose concentration
β_0	0.30	18.47
β_1	0.11	7.61
β_2	0.12	6.95
β_3	−0.04	11.08
β_4	0.06	3.64
β_5	0.05	3.75
β_6	0.01	0.84
β_7	0.04	2.46
β_8	0.02	6.35
β_9	−0.02	3.68

explained by the model was 88% for the conversion to glucose (mean squares of pure error: 0,001) and 91.4% for glucose concentration (mean squares of pure error: 4,2).

It should be stressed that for both response variables, the quadratic model fits the experimental data very appropriately as confirmed by the values of the correlation coefficient (0.91 for glucose concentration and 0.88 for conversion to glucose). The models generated in statistic analysis are represented by a quadratic function (Eq. 1) and the coefficient values are shown in the Table 2.

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 \cdot (\text{Temperature}) + \beta_2 \cdot (\text{Enzyme concentration}) \\ & + \beta_3 \cdot [\text{Solid}(\%)] + \beta_4 \cdot (\text{Temperature})^2 \\ & + \beta_5 \cdot (\text{Enzyme concentration})^2 \\ & + \beta_6 \cdot [\text{Solid}(\%)]^2 + \beta_7 \cdot (\text{Temperature} \times \text{Enzyme concentration}) \\ & + \beta_8 \cdot [\text{Temperature} \times \text{Solid}(\%)] \\ & + \beta_9 \cdot [\text{Solid}(\%) \times \text{Enzyme concentration}] \end{aligned} \quad (1)$$

Three optimizations were performed. First, the conditions for maximizing conversion of *celluligninG* to glucose were determined. Second, the conditions for maximizing glucose concentration in the enzymatic hydrolysis were established. The third optimization allowed the definition of values that maximize both variables simultaneously by means of Derringer and Suich function or global *desirability* function *(20,21)*.

Figure 4 shows the response surfaces generated in the simultaneous optimization of both response variables, conversion of *celluligninG* to glucose and glucose concentration. The best results are restricted to temperature ranges between 40 and 50°C, enzyme loading between 18 and 30 FPU/g *celluligninG*, and solid percentage between 8 and 10%, being the last factor, which presents the narrowest range of values that generated the zone of high values of the *desirability* function.

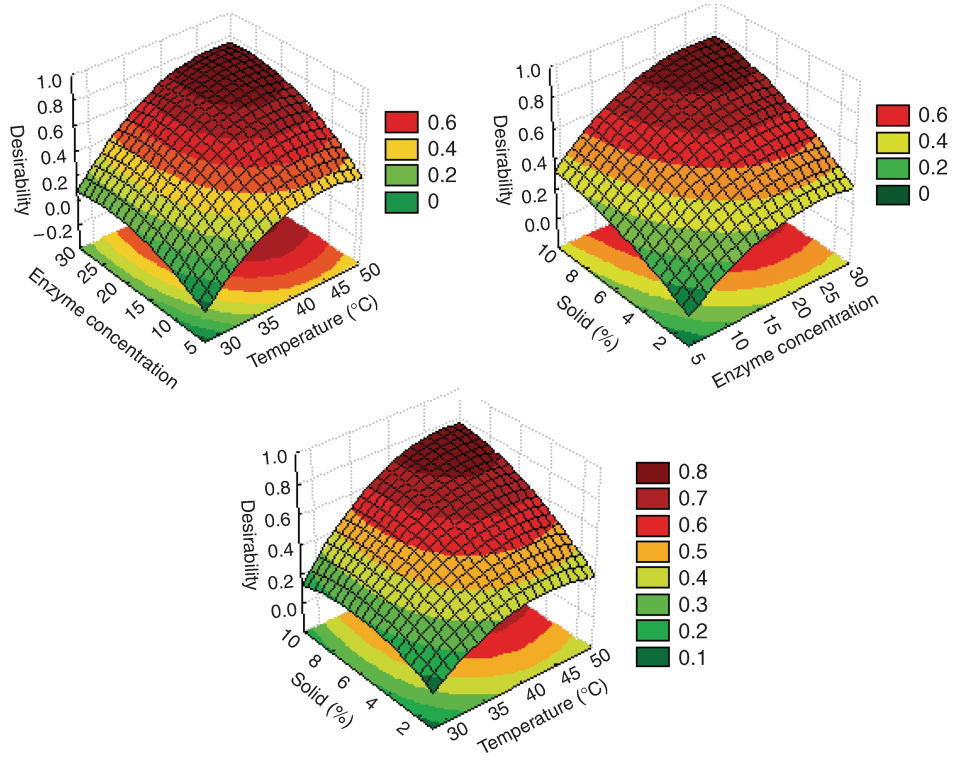

Fig. 4. Response surface for *desirability* function.

The optimal values for the response variables, glucose concentration, and conversion to glucose, and the optimal values of the *desirability* function are presented in Table 3. The conditions that lead to maximum values of conversion from *cellulignin*G to glucose and glucose concentration are similar for temperature and enzyme load. However, for the solid percentage variable the behavior differs, i.e., it is indicated the upper level (10%) for maximum glucose concentration and the use of its lower level (2%) for conversion to glucose. This makes the solid percentage variable as a decisive factor to reach significant values of both response variables. With the aim of evaluating the fermentability of the hydrolyzate obtained from *cellulignin*G arising from sugarcane bagasse, and in order to validate the optimal value predicted by the statistical analysis, the enzymatic hydrolysis was experimentally carried out, and the hydrolyzate was further fermented.

The validation results for the enzymatic hydrolysis were better than those predicted by the model (Table 3). This can be ascribed, probably, to the fact that these validation experiments were performed by using a bioreactor that allowed a better control of the process variables. Conversions of *cellulignin*G to glucose of 67% and glucose concentrations around 60.0 g/L were reached experimentally. According to the results obtained, high concentrations of glucose in the medium cannot be expected

Table 3
Predict Values to Statistical Model and Experimental Validations

				Predict values		Validation	
Optimal Points	Temperature (°C)	Solid (%)	Enzyme loading (FPU/g)	Conversion to glucose (g glucose/g *celluligninG*)	Glucose concentration (g glucose/L)	Conversion to glucose (g glucose/g *celluligninG*)	Glucose concentration (g glucose/L)
For conversion to glucose	43	2	24.4	0.58 ± 0.06	–	0.67 ± 0.09	13.05 ± 3.2
For glucose concentration	47	10	25.6	–	50.98 ± 2.9	0.52 ± 0.09	58.40 ± 3.2
For *desirability* function	47	10	25.9	0.47 ± 0.06	50.98 ± 2.9	0.54 ± 0.09	60.08 ± 3.2

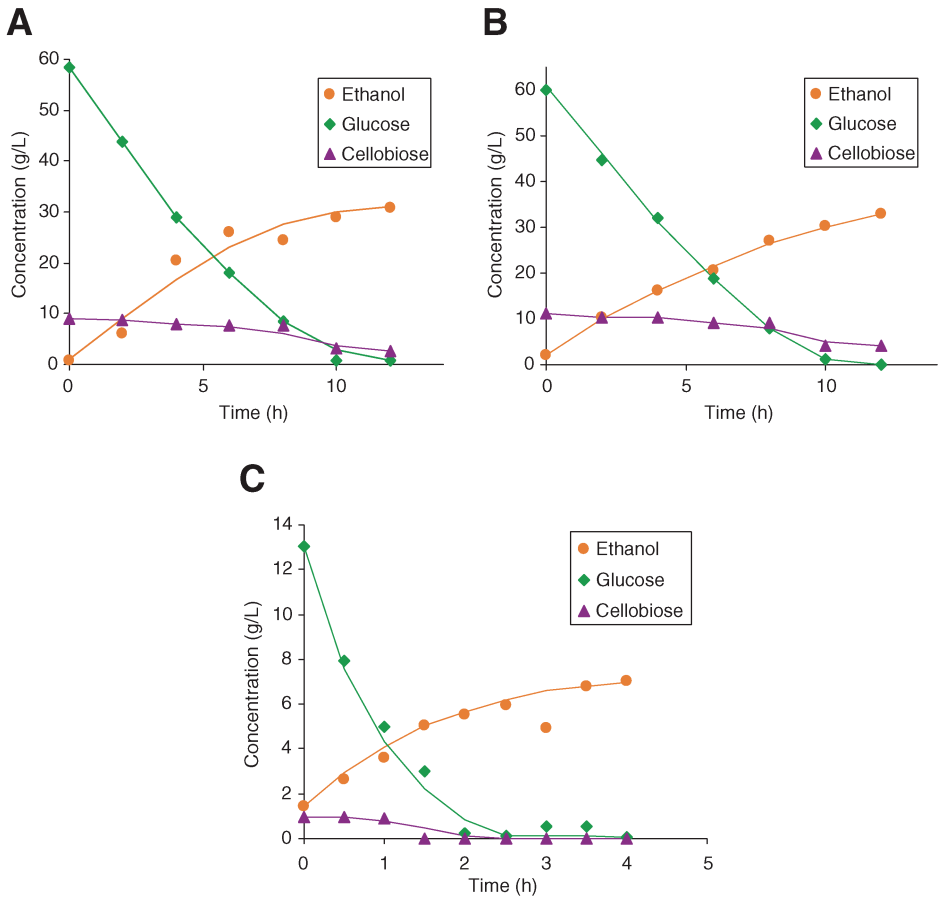

Fig. 5. Fermentation kinetics of hydrolyzates obtained by predicted conditions for high-glucose concentration **(A)** for *Desirability* function **(B)**, and for high conversion to glucose **(C)**.

because the conditions for high conversion to glucose are achieved in low solid percentage. The fermentations were carried out by using the hydrolyzate medium containing the hydrolysis residual solid, obtained under the conditions predicted by the statistical model. The fermentation kinetic profiles were obtained and presented in Fig. 5, evidencing the high fermentability of the hydrolyzate medium. Their behaviors are similar to those obtained using glucose synthetic medium (data not shown), evidencing the high fermentability of the hydrolyzate medium.

The similarity of the kinetic profiles of the Fig. 5A, b is owing to the analogous conditions used in the optimization process. For all cases the final ethanol concentration exceeded the theoretical conversion to ethanol considering the initial glucose concentration. This can be explained by the reactivation of the cellulolytic complex enzymes, particularly β-glucosidase, after the consumption of the glucose by the yeast. Thus, diminishing the inhibitory effect of this sugar on the enzymatic action, typical characteristic

of the *simultaneous saccharification and fermentation process*. A reduction of cellobiose concentration is detected after 10 h of fermentation, confirming the enzymatic reactivation.

Conclusions

The quadratic model generated in the statistical analysis fits adequately the behavior of the response variables (glucose concentration and conversion of *celluligninG* to glucose), in the enzymatic hydrolysis process when the factors (temperature, enzyme loading, and solid percentage) are varied within specified ranges. The conditions of hydrolysis that yielded the highest glucose concentration, 58.40 g/L, were: temperature 47°C, enzyme loading 25.6 FPU/g *celluligninG*, and solid percentage 10%. As far as the conversion of *celluligninG* to glucose, the highest value, 67%, was obtained in the following conditions: temperature 43°C, enzyme loading 24.4 FPU/g *celluligninG*, and solid percentage 2%. The *Desirability* function provided conditions in which both response variables were simultaneously optimized. These conditions were: temperature 47°C, enzyme loading 25.9 FPU/g *celluligninG*, and solid percentage 10%. The pH variable, in the range 5–6, does not present an important influence on the behavior of the enzymatic hydrolysis for any response variables (glucose concentration and conversion of *celluligninG* to glucose).

It is possible to conclude that the hydrolyzate produced in the enzymatic hydrolysis of *celluligninG* is easily fermented by *S. cerevisiae* yeast for the production of ethanol, resulting in a final ethanol concentration of 30.0 g/L, in only 10 h of fermentation. This provides a volumetric productivity value of 3.0 g/L.h, which is not so far from the values obtained in the conventional ethanol fermentation of sucrose in Brazil (5.0–8.0 g/L·h). The fermentation of the hydrolyzate of *celluligninG*, coming from the acid pretreatment of sugar-cane bagasse, stands as an excellent alternative for the production of fuel ethanol from lignocellulosic residues.

Acknowledgments

The authors acknowledge The Brazilian Oil Company (PETROBRAS) and the Brazilian Council for Research and Postgraduate Studies (CNPq) for financial support. They are also in debt with the Consulting Enterprise of Brazilian Distilleries-FERMENTEC, which dealt with the transportation of sugarcane bagasse from São Paulo State to the Laboratory of Bioprocess Development at the Federal University of Rio de Janeiro.

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