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Lime Pretreatment of Sugarcane Bagasse for Bioethanol Production

Sarita C. Rabelo · Rubens Maciel Filho · Aline Carvalho Costa

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Abstract The pretreatment of sugarcane bagasse with lime (calcium hydroxide) is evaluated. The effect of lime pretreatment on digestibility was studied through analyses using central composite design (response surface), considering pretreatment time, temperature, and lime loading as factors. The responses evaluated were the yield of glucose from pretreated bagasse after enzymatic hydrolysis. Experiments were performed using the bagasse as it comes from an alcohol/sugar factory (non-screened bagasse) and bagasse in the size range from 0.248 to 1.397 mm (screened bagasse) (12-60 mesh). It was observed that the particle size presented influence in the release of fermentable sugars after enzymatic hydrolysis using low loading of cellulase and β-glucosidase (3.5 FPU/g dry pretreated biomass and 1.0 IU/g dry pretreated biomass, respectively).

Keywords Lignocellulose materials · Sugarcane bagasse · Pretreatment · Lime · Enzymatic hydrolysis · Statistical analysis

Introduction

In recent years, efforts have increased toward commercial production of ethanol, considered the most promising biofuel from renewable resources, and it is well known that a low-cost feedstock is a very important factor in establishing a cost-effective technology [1].

The production of ethanol from any lignocellulosic biomass generally involves four process steps—feedstock pretreatment, enzymatic saccharification, fermentation, and ethanol recovery [2].

Pretreatment is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars [3]. Costs are due to the use of steam

S. C. Rabelo · R. M. Filho · A. C. Costa (✉)
School of Chemical Engineering, University of Campinas, UNICAMP, Campinas, Brazil
e-mail: accosta@feq.unicamp.br

and chemical products and the need for expensive corrosion resistant reactors. Pretreatment has a great potential for improvement of efficiency and lowering of costs through research and development [4–7].

Of the two possible hydrolysis methods, acid and enzymatic hydrolysis, acid hydrolysis is efficient and relatively inexpensive, but it forms compounds that might seriously inhibit the subsequent fermentation. On the other hand, enzymatic hydrolysis has been improving along with the development of biotechnology and, therefore, there is a possibility of high improvements of efficiency and costs. It is expected that improvements in thermostability and in the cellulose-binding domain of cellulase will lead to a more than tenfold increase in enzyme performance in the decade ahead [8]. Achievement of this perspective will enhance economic competitiveness of the enzymatic hydrolysis as an approach for utilizing lignocellulosic biomass.

In order to hydrolyze lignocellulosic biomass with enzymes successfully, it is very important to use a suitable pretreatment, because crystallinity of cellulose, degree of polymerization (DP), moisture content, available surface area, and lignin content are factors that hinder the attack of enzymes [9].

Certain kinds of chemical, physical, and/or biological pretreatments remove lignin, reduce the degree of cellulose crystallinity, and increase the surface area of biomass, resulting in an enhancement of lignocellulosic substrate digestibility. Some chemical pretreatments are considered as especially promising approaches because of their high reactivity under moderate conditions such as room temperature and normal pressure.

An effective pretreatment is characterized by several criteria. It should avoid the need for reducing the size of biomass particles, preserve the pentose (hemicellulose) fractions, limit formation of degradation products that inhibit growth of fermentative microorganism, minimizes energy demands, and limit costs. Also, the pretreatment agent should have low cost and inexpensive recycle [5].

Lime (calcium hydroxide) pretreatment has regained interest as one of the promising pretreatment technologies being studied. This pretreatment has low formation of fermentation inhibitors, increases pH, and provides a low-cost alternative for lignin solubilization, removing approximately 33% of lignin and 100% of acetyl groups. The action of lime is slower than other pretreatments but its low cost and safe handling make it attractive [6].

In this work, the pretreatment of sugarcane bagasse with lime [10–19] is evaluated. It was chosen for occurring in mild conditions (temperature, pressure, and absence of acids), besides being an alkaline process, which is expected to cause less sugar degradation than acid processes [18]. Also, lime is an inexpensive reagent and can be easily recovered as calcium carbonate by neutralization with carbon dioxide, although this is an energy-intensive process and not yet economically feasible. The calcium hydroxide can be subsequently regenerated using the established lime kiln technology [18].

The effect of lime pretreatment on digestibility was studied through analyses using central composite design (response surface), considering pretreatment time, temperature, and lime loading as factors. The responses evaluated were the yield of total reducing sugars (TRS) and of glucose from pretreated bagasse after enzymatic hydrolysis. Experiments were performed using the bagasse as it comes from an alcohol/sugar factory (non-screened bagasse) and bagasse in the size range from 0.248 to 1.397 mm (screened bagasse) (12-60 mesh). The objective was to evaluate the influence of particle size and the possibility of using the bagasse as it comes from the mills.

Materials and Methods

Substrate

Fresh sugarcane bagasse was obtained from the sugar plant Usina São Luiz-Dedini S/A, (Pirassununga/SP, Brazil). It was dried at 45 °C for 48 h, left for 48 h at room temperature, put into plastic bags, and kept in a freezer. The dry matter content of the bagasse after being dried was 95%. The bagasse used in the tests was divided into two parts. One part came from the mill, without prior screening and presented highly heterogeneous particle sizes. This part will be called non-screened bagasse throughout this article. The other part was screened in the size range of 0.248 to 1.397 mm (12-60 mesh). Smaller particles were discarded because they corresponded mainly to sand. A granulometric analysis of the bagasse is shown in Fig. 1.

Chemical Analysis of Bagasse Samples

Samples of the screened and non-screened bagasse were milled to pass through a 0.75-mm screen. Approximately 4 g of milled sample was extracted with 95% ethanol for 6 h in a Soxhlet apparatus [20]. Ash content was determined after burning of the samples in a muffle at 500 °C for 4 h [21]. Extracted bagasse samples were hydrolyzed with 72% sulfuric acid at 30 °C for 1 h (300 mg of sample and 3 ml of sulfuric acid). The acid was diluted to a final concentration of 4% (addition of 84 ml of water) and the mixture heated at 125 °C/1 atm for 1 h [22]. The residual material was cooled and filtered through porous glass filter number 3. The solids were dried to constant weight at 105 °C and determined as insoluble lignin. The soluble lignin concentration in the filtrate was determined by

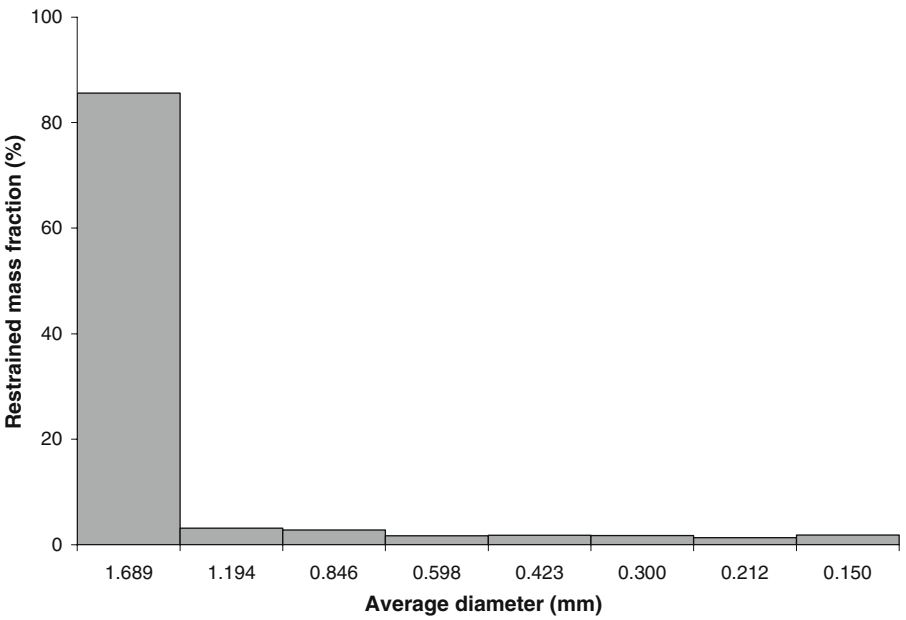

Fig. 1 Percentage of the restrained mass fraction versus average diameter of the particle

measuring absorbance at 205 nm and using the value of 105 l g^{-1} cm^{-1} as the absorptivity of soluble lignin [23]. The concentrations of monomeric sugars in the soluble fraction were determined by high performance liquid chromatography (HPLC) using a Biorad HPX87H column at 45 °C, eluted at 0.6 ml/min with 0.005 mol/l sulfuric acid. Sugars were detected in a 30 °C temperature-controlled RI detector (Knauer HPLC pump and detector). In these conditions, xylose, mannose, and galactose eluted at the same retention time and were integrated as a single peak. Glucose, xylose, arabinose, and acetic acid were used as external calibration standards. Sugar loss by acid degradation was considered using the Sugar Recovery Standards as suggested by NREL method. The factors used to convert sugar monomers to anhydromonomers were 0.90 for glucose and 0.88 for xylose and arabinose. Acetyl content was calculated as the acetic acid content multiplied by 0.7. These factors were calculated based on water addition to polysaccharides during acid hydrolysis [24–27]. Table 1 shows the composition of the screened and non-screened bagasse.

Pretreatment

The pretreatment agent evaluated was lime (calcium hydroxide). The influence of pretreatment time, temperature and lime loading on the performance of the pretreatment was evaluated during the experiments. Non-screened bagasse (4 g) and screened bagasse (4 g) were treated with 100 ml of the pretreatment solution in 500 ml flasks in an orbital shaker (Marconi MA-832) agitated at 150 rpm. Pretreatment agent was prepared by adding $Ca(OH)_2$ in distilled water.

Lime Consumption

Lime was used as the sole alkali to pretreat bagasse. The amount of lime consumed depended on the pretreatment conditions such as temperature and time. Lime consumption was determined by titration using a solution of chlorohydric acid (HCl) 5.0 mol/l to determine the remaining amounts of lime in the treated biomass mixture. The amounts of lime consumed during the pretreatment at each condition were determined by pH neutralization with a standard solution of acid. Figure 2 show the lime load added and the unreacted load after each one of the assays for the non-screened and screened bagasse.

Enzymatic Hydrolysis

After pretreatment, the substrate was washed to remove soluble matter, dried, and weighted to measure mass loss. The present market offers many cellulase preparations (including those obtained from *Trichoderma reesei*) containing low levels of β-glucosidase, which

Table 1 Composition of the non-screened and screened sugarcane bagasse.

	Non-screened bagasse %	Screened bagasse %
Glucan	39.6±0.9	34.1±0.9
Xylan	19.7±0.5	17.7±0.5
Arabinan groups	1.7±0.1	2.0±0.1
Acetyl groups	2.5±0.1	2.4±0.1
Lignin	25.8±1.6	29.3±1.6
Extractives	2.3±0.1	2.3±0.1
Ash	3.8±0.1	5.3±0.1

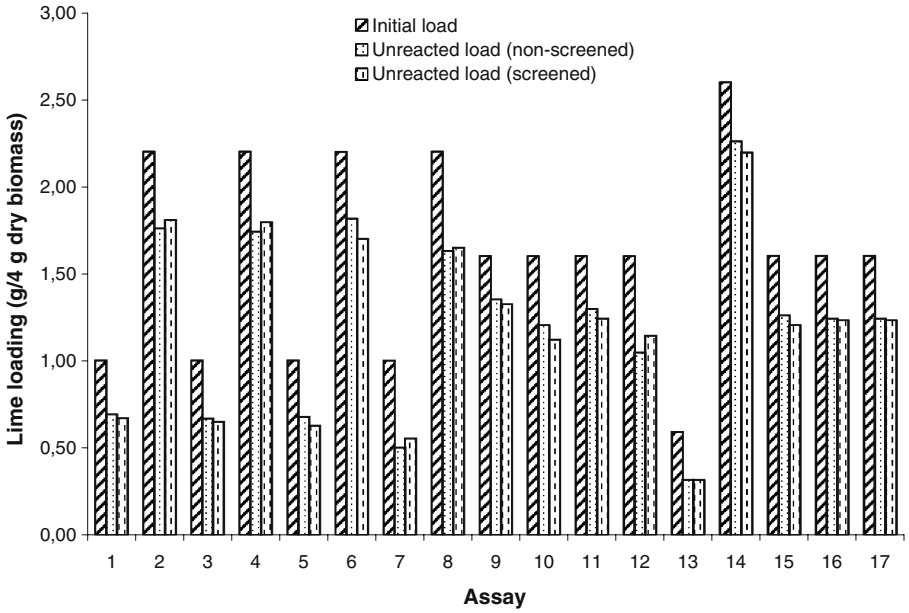

Fig. 2 Lime load initial added and unreacted load in each one of the conditions of the assays

leads to an increased accumulation of cellobiose in the enzymatic hydrolyzates of the cellulose. The inability of industrial glucose-fermenting yeasts to ferment cellobiose results in incomplete conversion of cellulose hydrolyzate to ethanol, significantly diminishing its final yield. These drawbacks may be overcome by supplementation of the cellulase complex with a β-glucosidase from other sources [28]. One gram of the pretreated bagasse was hydrolyzed with 300 ml of solution containing cellulase and β-glucosidase with pH adjusted to 4.8. Enzymatic hydrolysis was performed with a commercially available cellulase produced by *Trichoderma reesei* (Sigma) and β-glucosidase from *Aspergillus niger* (Sigma) with concentrations of 3.5 FPU/g dry pretreated biomass and 1.0 IU/g dry pretreated biomass, respectively, were used. The enzymes were solubilized in a 50 mM citrate buffer (pH 4.8).

Hydrolysis experiments were carried out in 500 ml flasks in orbital shaker (Marconi MA-832) agitated at 100 rpm at 50 °C. Aliquots were taken periodically, boiled to deactivate the enzymes, and analyzed for glucose and reducing sugars.

Enzymatic Activities

Cellulase activity was determined as filter paper units per milliliter, as recommended by International Union of Pure and Applied Chemistry [29, 30]. β-Glucosidase activity was determined through a solution of cellobiose 15 mmol/l and expressed in units per milliliter (IU/ml) [31]. Enzyme activity was 64.11 FPU/ml for cellulases and 308.37 IU/ml for β-glucosidase.

Analytical Methods

Glucose yield was measured using a kit based on the glucose oxidase reaction (GOD-PAP, Laborlab) and TRS yield was determined by the dinitrosalicylic acid (DNS) method

[32]. In both methods, the standard glucose (Merck) was used for the preparation of standard curve.

Experimental Design

A central composite design (response surface) of three factors at three levels was developed with three replicates in the central point [33]. The experimental design matrix is shown in Table 2. 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 *F* tests [33].

The values of glucose and TRS yields used for the statistical analysis were picked at the reaction time after which no significant changes in these variables were detected. 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 STATISTICA 7.0 (Statsoft, Inc., Tulsa, OK, USA) software was used here in order to implement all these statistical analysis.

Results and Discussion

The results of experiments obtained by utilizing a central composite with three replicates in the central point were analyzed by considering glucose and TRS yields after hydrolysis of pretreated bagasse for the non-screened (NS) and screened (S) samples for the pretreatment with lime as output (response) variables. The glucose and TRS yields were expressed as milligram per gram of dry raw bagasse (not pretreated). Table 3 shows the design matrix where the maximum TRS and glucose yield obtained for each assay are marked in bold. The ranges of the factors for the pretreatment were chosen based on literature [10–19].

It can be seen from Table 3 that, in the operational conditions used in this work, maximum TRS release was obtained with non-screened bagasse (392.8 mg/g dry non-screened bagasse); thus, when all the reducing sugars (hexoses and pentoses) are of interest, non-screened bagasse is a better choice, which reduces substantially the costs of the process. As the industrial fermenting microorganisms used nowadays for industrial ethanol production do not ferment pentoses, in many practical applications, the product of interest may be glucose. In relation to the glucose yield, maximum was obtained with screened bagasse (218.0 mg/g dry screened bagasse).

Figure 3 shows the mass resulting after pretreatment of 4 g of non-screened and screened bagasse samples for each of the essays described in Table 3. From Fig. 3, it can be observed that mass loss is greater for the screened samples. According to Mosier et al. [5],

Table 2 Coded factor levels and real values lime pretreatment.

	Levels				
Factors	−1, 41	−1	0	+1	+1, 41
Pretreatment time (h)	8.4	20	37	54	65.6
Temperature (°C)	53.2	60	70	80	86.8
Lime loading (g/g dry biomass)	0.15	0.25	0.40	0.55	0.65

Table 3 Design matrix presenting mass of TRS and glucose released after hydrolysis of lime pretreated bagasse (non-screened—NS and screened—S).

Assay	Time (h)	Temperature (°C)	Lime loading (g/g)	TRS (NS) (mg/g)	Glucose (NS) (mg/g)	TRS (S) (mg/g)	Glucose (S) (mg/g)
1	20	60	0.25	272.4	155.1	303.6	182.6
2	20	60	0.55	245.8	143.0	321.3	179.1
3	20	80	0.25	281.4	181.8	310.6	192.0
4	20	80	0.55	332.3	174.0	339.8	210.8
5	54	60	0.25	334.7	185.7	349.6	203.4
6	54	60	0.55	331.6	179.5	333.5	201.8
7	54	80	0.25	**392.8**	202.2	326.5	206.2
8	54	80	0.55	358.6	192.6	316.3	195.5
9	8.4	70	0.40	235.5	142.1	292.2	177.4
10	65.6	70	0.40	350.9	**203.2**	**367.2**	**218.0**
11	37	53.2	0.40	307.2	167.0	315.5	191.1
12	37	86.8	0.40	375.7	201.2	362.8	213.3
13	37	70	0.15	284.0	164.8	323.9	194.9
14	37	70	0.65	277.5	172.2	336.1	204.8
15	37	70	0.40	323.6	189.1	355.0	199.0
16	37	70	0.40	330.4	182.7	349.3	206.0
17	37	70	0.40	341.7	185.8	348.9	203.0

pretreatment with lime has a major effect in delignification and in increasing accessible surface area, but its effect in removing hemicellulose is minor.

The results of this analysis are shown by using the Pareto charts as they present, very clearly, the most significant effects. In these charts, the effect estimates divided by their standard errors are sorted from the largest absolute value to the smallest absolute value. The magnitude of each effect is represented by a column and a line going across the columns indicates how large an

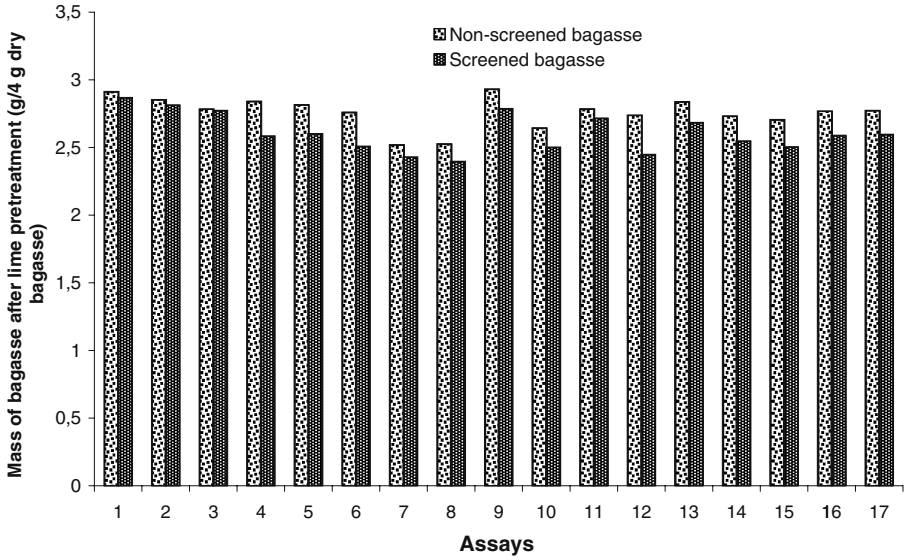

Fig. 3 Mass of bagasse after pretreatment of 4 g of non-screened and screened samples bagasse

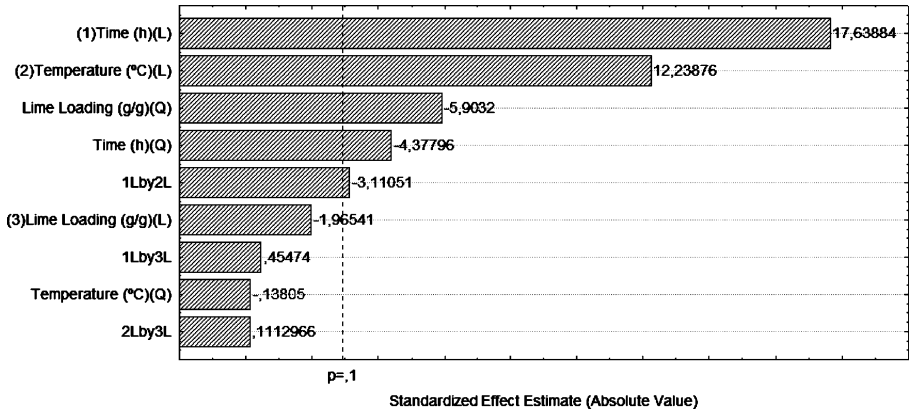

Fig. 4 Pareto chart of standardized effects for the glucose yield (mg/g raw non-screened bagasse)

effect must be to be considered statistically significant. In this work, the vertical line corresponds to a p value of 0.1, which implies in a 90% level of significance [33]. Figures 4 and 5 shows the Pareto chart of standardized effects for glucose yield after enzymatic hydrolysis of pretreated non-screened and screened samples of bagasse, respectively.

In the Pareto chart for glucose yield of the non-screened bagasse (Fig. 4), the largest effect is the linear effect of pretreatment time, followed by the linear effect of temperature, both affecting positively the glucose yield. In general, the interaction factors have less significant effects over the conversion to glucose and only the interaction between time and temperature is significant. The only effect involving lime loading that is significant is the quadratic effect, and it is negative, indicating that it is not necessary to add great amounts of lime. Figure 2 shows that lime is not completely consumed even in the lowest lime loading considered (0.15 g/g, corresponding to assay 13).

The Pareto chart for glucose yield of the screened bagasse is shown in Fig. 5. The highest effects are also the linear effects of time and temperature, both positive. The only other significant effect is the interaction between pretreatment time and temperature.

Table 4 depicts the analysis of variance (ANOVA) for the models of glucose yield after hydrolysis for lime pretreatment of non-screened and screened bagasse when only the

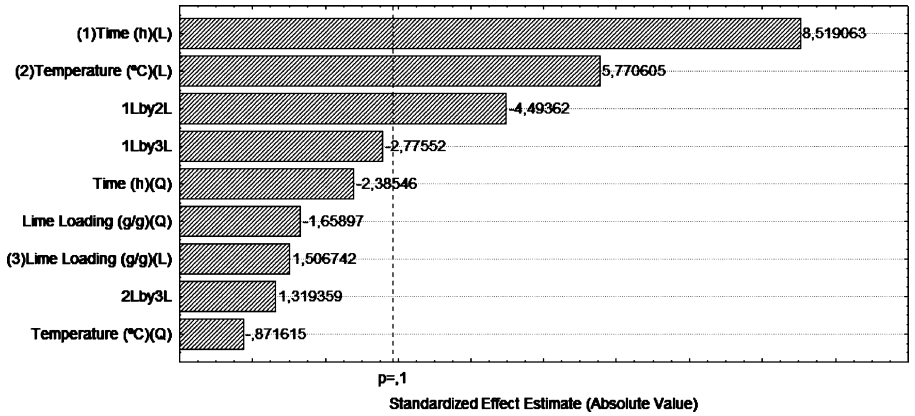

Fig. 5 Pareto chart of standardized effects for the glucose yield (mg/g raw screened bagasse)

Table 4 ANOVA for the models describing glucose yield from non-screened (NS) and screened bagasse.

Source of variation	Sum of squares (SQ)		Degrees of freedom (DF)		Mean square (MS)		*F* value	
	Glu (NS)	Glu (S)	Glu (NS)	Glu (S)	Glu (NS)	Glu (S)	Glu (NS)	Glu (S)
Regression (R)	5,324.9	1,554.8	5	3	1,065.0	518.3	37.33*	11.29*
Residual (r)	313.8	596.7	11	13	28.5	45.9		
Lack of fit (Lf)	293.3	572.0	9	11	32.6	52.0	3.17**	4.22**
Pure error (Pe)	20.5	24.7	2	2	10.3	12.3		
Total (T)	5,638.8	2,151.5	16	16				
R^2	0.996	0.989						
F listed values (90% of confidence)							$*F_{5,11}$=2.45 $**F_{9,2}$= 9.38	$*F_{3,13}$=2.56 $**F_{11,2}$= 9.40

*F test for statistical significance of the regression=MS_R/MS_r. **F test for lack of fit=MS_{Lf}/MS_{Pe}

significant coefficients are taken into account. It can be seen that the models present high correlation coefficient and can be considered statistically significant with 90% of confidence according to the F test, as they presented calculated F values greater than the listed ones [33]. Also, they do not present evidence of lack of fit, as the calculated values for the F test for lack of fit are much smaller than the listed values. Thus, the quadratic models proposed fit the experimental data accurately. They are represented by Eqs. (1) and (2).

$$\text{Glucose yield (mg/g of dry non–screened bagasse)} = 185.6 + 15.3 \cdot t - 4.1 \cdot t^2 + 10.6 \cdot T - 5.6 \cdot L^2 - 3.5 \cdot t \cdot T \quad (1)$$

$$\text{Glucose yield (mg/g of dry non–screened bagasse)} = 198.8 + 8.1 \cdot t + 5.5 \cdot T - 5.6 \cdot t \cdot T \quad (2)$$

In these equations, t, T, and L are the coded values of pretreatment time, temperature, and lime loading, respectively.

The proposed models can be used to plot response surfaces and for prediction or optimization purposes. The response surface for glucose yield from non-screened bagasse is

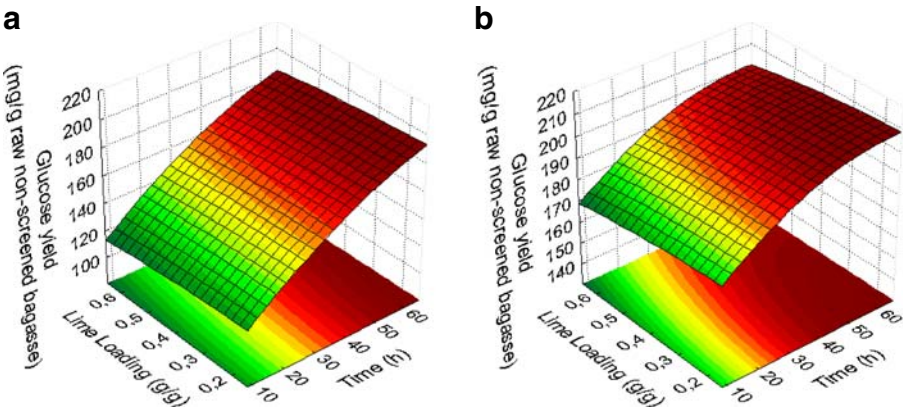

Fig. 6 Glucose yield from non-screened bagasse. **a** Temperature 53.2 °C. **b** Temperature 83.8 °C

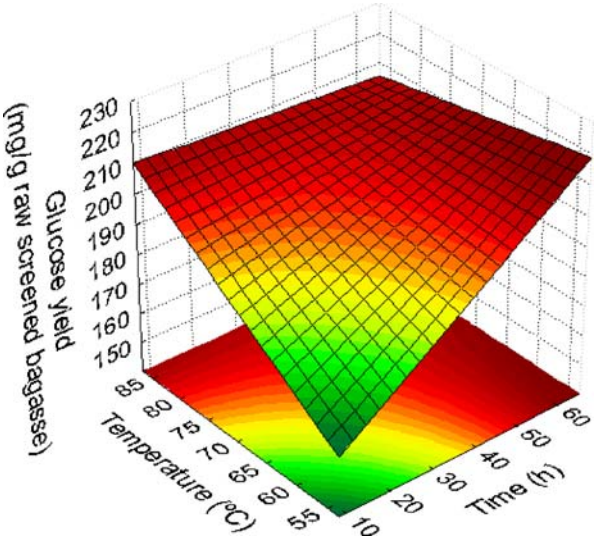

Fig. 7 Glucose yield from screened bagasse

depicted in Fig. 6. Figure 6a shows glucose yield versus lime loading and pretreatment time when temperature is 53.2 °C, the lowest temperature considered, and Fig. 6b shows the same response surface when temperature is 83.8 °C, the highest temperature considered. From these figures, it can be seen that the highest glucose yields are obtained with high pretreatment time and low lime loading, although lime loading shows a low influence in the response, with high yields being obtained for high pretreatment times in all the lime loading range considered.

Maximum glucose yield is obtained with high temperature, high pretreatment time, and low lime loading (Fig. 6b), but the small difference between the maximum glucose yield obtained in Figs. 6a and b indicates that the influence of temperature is not very strong. The strongest influence is of pretreatment time.

The response surface for glucose yield from screened bagasse is shown in Fig. 7. It can be seen that maximum glucose yield was obtained in two regions: low temperature and high pretreatment time or high temperature and low pretreatment time. As lime loading was not significant at the 90% confidence level, it can be maintained in the minimum value.

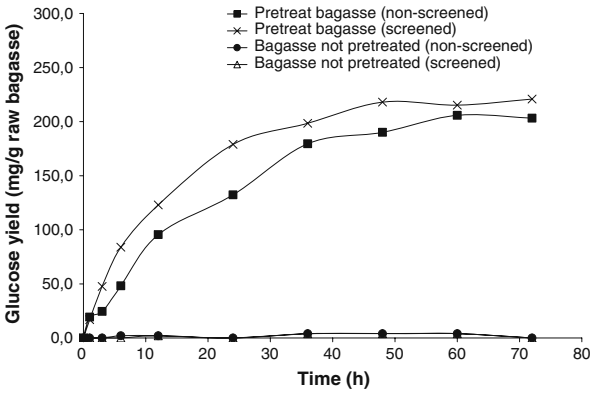

Fig. 8 Glucose yield from enzymatic hydrolysis of bagasse in the best conditions

Figure 8 shows the best experimental hydrolysis profiles for screened and non-screened bagasse pretreated with lime (assays 10) and the hydrolysis profiles for bagasses without pretreatment. The importance of the stage of pretreatment in the process can be observed as glucose for the bagasse not pretreated is practically inexistent.

Conclusion

The effectiveness of lime pretreatment in improving sugarcane bagasse susceptibility to enzymatic hydrolysis was evaluated. The effect of lime pretreatment on digestibility was studied through analyses using central composite design (response surface), considering pretreatment time, temperature and lime loading as factors. The performance was evaluated by the release of glucose after hydrolysis of the pretreated bagasse.

The influence of screening the bagasse before pretreatment in hydrolysis performance was assessed. All the tests were performed using bagasse as it comes from a sugar/alcohol factory and bagasse screened with particles between 0.248 and 1.397 mm. The tests performed have shown that the performance of the hydrolysis of bagasse pretreated with lime in the range of conditions studied is better for screened bagasse if we are interested in glucose. Results for non-screened bagasse suggest that, for maximum yield of glucose, pretreatment time and temperature should be increased and the lime loading diminished, so pretreatment should be performed for 65.6 h at 86.8 °C with lime loading of 0.15 g/g biomass for high glucose yield.

For screened bagasse, two conditions lead to high glucose yield: high pretreatment time, low temperature, and low lime loading or high temperature, low pretreatment time, and low lime loading.

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