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Dilute Acid Pretreatment Optimization from Sugarcane Wastes by Experimental Design

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----------ABSTRACT----------

The pretreatment (PT) means an important step for 2G ethanol production from lignocellulosics wastes. Therefore, it is necessary that this stage provides feasible results to favour the hydrolysis and fermentations steps. Taking this into account, the aim of this work was evaluate, between the type of inorganic acids (sulfuric, phosphoric, hydrochloric and nitric), the one which can promote the highest total reducing sugars (TRS) releasing on PT step from straw and sugarcane bagasse. In addition, a blend, in a straw/bagasse ratio of 1:1, 1:3 and 3:1 was submitted to PT, applying the same acids reported previously. Lastly, after the choice of acid and straw/bagasse ratio to be applied in the assays, an experimental design was performed, aiming the optimization of PT conditions. Factors evaluated were acid concentration (%), time (min) and solids loading (%). The best condition from experimental design concerning TRS concentration was conducted to enzymatic hydrolysis and fermentation. The finds showed that sulfuric acid outperformed the others. At the end of the experiments, 25.59 g.L^{-1} of ethanol concentration was obtained.

Keywords: *Lignocellulosic feedstocks, optimization, pretreatment, response surface methodology.*

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I. INTRODUCTION

Lignocellulosic materials have been widely studied around the word for second generation ethanol production [1,2,3]. Particularly in Brazil, sugarcane wastes are promising towards replacing fossil fuels, since this country is the world's largest producer of sugarcane. Sugarcane bagasse can be used in several applications, such as energy cogeneration and production of ethanol and animal feed. Sugarcane straw is used for land cover or can be burned in industries to supply energy [4,5]. Both straw and sugarcane bagasse are mostly composed of cellulose, hemicellulose and lignin. Since these residues present a hard structure, a pretreatment step is mandatory to disrupt this recalcitrance [6]. During an acid pretreatment, hydronium-catalyzed reactions promote the degradation of the hemicellulosic fraction, on the other hand the cellulose and lignin remain almost totally unchanged [7]. Several acid solutions have been aplyied in the literature for this purpose, such as sulfuric, maleic, oxalic, hydrochloric, phosphoric and nitric [8,9]. Dilute acids are particularly interesting due to be efficient in releasing fermentable sugars and minimizing problems with corrosion. Hydrolysis and fermentation are the sequencial steps for 2G ethanol production process and its success it is closely correlated with the results

reached in the pretreatment [10,11]. Taking all this into account, this work presents a systematic study concerning the pretreatment step for 2G ethanol production from straw and sugarcane bagasse. The performances of sulfuric, phosphoric, nitric and hydrochloric acids were compared. Straw and sugarcane bagasse were applied in the assays separately and mixed, in the straw/bagasse ratio of 1:1, 1:3 and 3:1. After the acid and ratio choice, an experimental design was conducted to determinate the optimum conditions for the total reducing sugars (TRS) production, with the variation of time (min), acid concentration (%) and solids loading (%). Lastly, enzymatic hydrolysis and fermentation were performed to the best condition indicated by experimental design.

II. MATERIALS AND METHODS

2.1 Raw material.

Bagasse and sugarcane straw used to carry out the experiments were kindly provided by Coruripe Mill, located in Coruripe, Alagoas, Brazil. These feedstocks were dried at room temperature until it was approximately 10% in moisture content. After, it was milled in a Willey type mill to a particle size of 20 mesh, put into plastic bags, and kept in a freezer (-8 °C) for the subsequent experiments.

2.2 Dilute acid pretreatment.

Firstly, four acids – sulfuric (H_2SO_4), phosphoric (H_3PO_4), hydrochloric (HCL) and nitric (HNO_3) – were applied in dilute acid pretreatment, to evaluate their performance. These assays were conducted applying straw and sugarcane bagasse separately, at 15 and 30 minutes and applying 0.5 and 1.0 % (w/w) of acid concentration. A second step was conducted the pretreatment experiments by using mixed straw and bagasse, in a straw/bagasse ratio of 1:1, 1:3 and 3:1. In this case, 0.5 and 1.0 % (w/w) of acid concentration was employed, however only the time of 15 minutes was evaluated. Lastly, after choosing the type of acid and straw/bagasse ratio to be employed in the assays, an experimental design (Statistica®, 7.0 version) was performed to obtain the best conditions concerning releasing total reducing sugars (TRS) during the pretreatment step. Factors evaluated were time (min), acid concentration (%) and solids loading (%). The evaluated response was TRS concentration. Levels chosen to each factor as showed in Table 1.

Table 1. Level of variables chosen.

Nº. Variable	Level Chosen	
	Low	High
1. Acid concentration (%)	0.25	0.75
2. Time (h)	5	15
3. Solids loading (%)	10	20

The lignocellulosic residues, separately or mixed, were submitted to a pretreatment in acid solution, in an autoclave at 121 °C. After returning to ambient temperature, the solid was then separated from the liquid fraction by filtration. Finally, solid fraction was washed with hot water (70 °C) to remove the solubilized contents adhered to the surfaces. All hydrolysis experiments were performed in triplicate.

2.3 Enzymatic hydrolysis.

After the analysis of results, the assay that presented the highest TRS concentration was conducted to enzymatic hydrolysis. The enzymatic complex applied in the experiments was Cellic®CTec2, donated by Novozymes Latin America (Araucária, Paraná, Brazil). This complex presented 203 $FPU.mL^{-1}$ of enzymatic activity [12]. Enzymatic hydrolysis was conducted with reaction volume of 30 mL, 150 rpm, 72 hours, at 50 °C, in sodium citrate buffer (50 mM; pH 4.8). This assays occurred at solids loading of 10 % (w/v), under batch strategy. Enzyme dosage was 15 $FPU.g^{-1}_{substrate}$. After hydrolysis assays, total reducing sugars (TRS) were determined by DNS spectrophotometer method [13].

2.4 Fermentation.

Cells of *Saccharomyces cerevisiae* were cultivated in Erlenmeyer flasks containing YPD medium (10 g/L yeast extract, 20 g/L peptone, 40 g/L glucose) in stirred shaker incubator at 30 °C and 150 rpm. After 48 h of cultivation, the medium was centrifugated (8,000 rpm, 10 min) and cells were resuspended in the hydrolyzed. Fermentation assays were conducted at 24 hours, 30 °C and 150 rpm.

III. RESULTS AND DISCUSSION

3.1 Effect of Dilute Acids Pretreatment on the Residues.

Figure 1 shows total reducing sugars concentrations released during the pretreatment, under several conditions (acid concentration and time) and applying four different acids solutions. These assays were conducted for sugarcane straw. The range of TRS concentration was 9.66-30.07 $g.L^{-1}$. It can be observed that, in general, phosphoric acid presented the lowest values, followed by hydrochloric acid. On the other hand, the highest TRS values were reached to sulfuric and nitric acids.

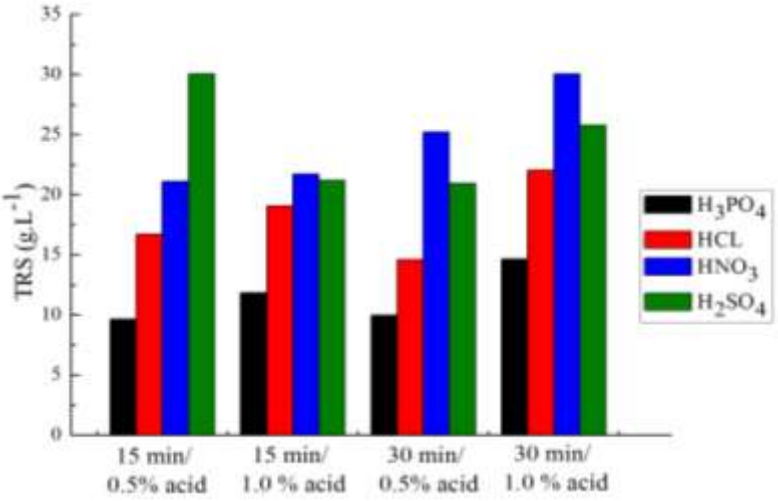

Figure 1. Total reducing sugars released during the sugarcane straw pretreatment step, under several conditions and applying four different acid solutions.

The literature affirms that between the acids employed in the pretreatment of biomass, phosphoric acid is a weak acid when compared to sulfuric acid. Therefore, phosphoric acid has been applied as a concentrated solution [14]. Another work presented in the literature, affirms that the optima condition for grass acid hydrolysis was 2.5 % phosphoric acid at a temperature of 175 °C, under 15 minutes [15]. These reported works applied more severe conditions than this study. In this way, TRS concentrations founded here, applying phosphoric acid, presented the lowest values. Several works has been employed dilute sulfuric acid because of its high catabolic activity, aiming the hemicellulosic fraction solubilization [9,16,17]. Rodríguez-Chong et al, [18] conducted a study of sugarcane bagasse hydrolysis using nitric acid and a comparison was performed with sulfuric and hydrochloric acids. HCl was the least efficient acid due to the large amount of inhibitors generated, whereas H_2SO_4 was little more efficient than HNO_3. Selecting only the experiments of industrial interest, nitric acid was the most efficient catalyst for hydrolysis. These results are in accordance with this work since the best TRS values were obtained applying nitric and sulfuric acid.

Similarly to sugarcane straw, Figure 2 shows total reducing sugars concentrations released during the sugarcane bagasse pretreatment, under several conditions and applying four different acid solutions.

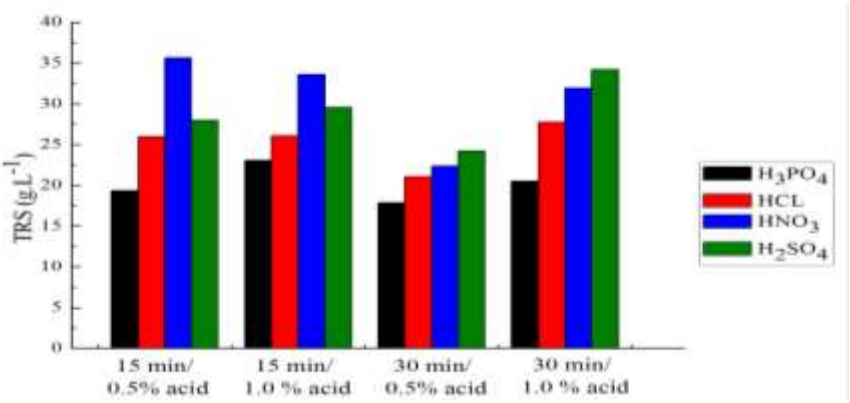

Figure 2. Total reducing sugars released during the sugarcane bagasse pretreatment step, under several conditions and applying four different acid solutions.

The range of TRS concentration was 17.84-35.65 $g.L^{-1}$. In the same way than straw results, phosphoric acid presented the lowest values, although higher than those showed by sugarcane straw. In addition, overall analysis showed that hydrochloric, nitric and sulfuric acids presented higher TRS concentrations than those obtained by sugarcane straw. The highest TRS values were reached to sulfuric acid (at 30 min, followed by nitric acid) and nitric acid (at 15 min, followed by sulfuric acid).

Since both straw and bagasse are from the same crop, these wastes can be applied as a blend, saving costs concerning transport and storage. In this way, an evaluation was conducted applying mixed sugarcane wastes, in a straw/bagasse ratio of 1:1, 1:3 and 3:1. Figure 3 presented the obtained results.

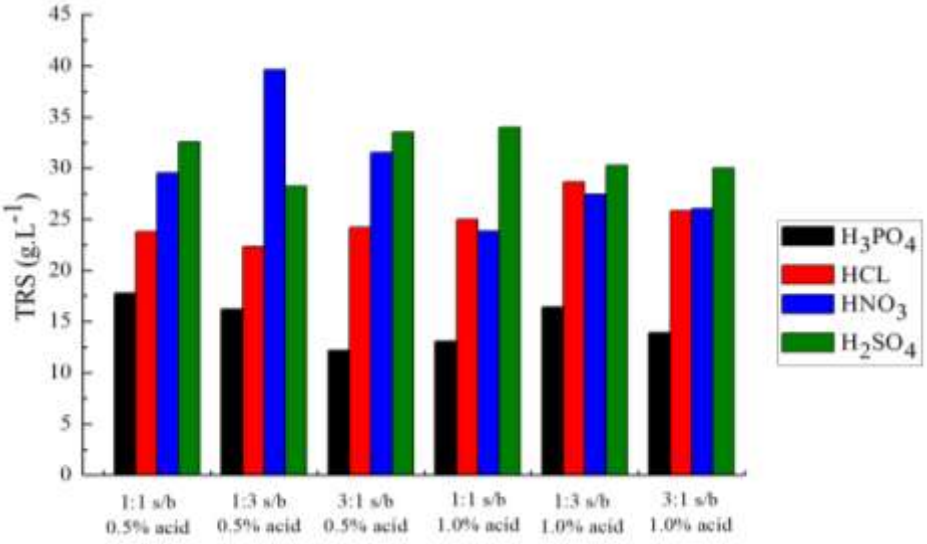

Figure 3. Total reducing sugars released during the pretreatment step (mixed straw and sugarcane bagasse), under several conditions and applying four different acid solutions.

From Figure 3, sulfuric acid achieved the higher TRS values for almost all evaluated conditions. Only one assay (straw/bagasse ratio of 1:3 and 0.5 % of acid) presented TRS nitric acid value more than sulfuric acid. Taking this into account, sulfuric acid was chosen to be applied in the experimental design conducted in the sequencial study. These assays were performed with a straw/bagasse ratio of 1:1, based on the results presented in Figure 3, considering the TRS from the sulfuric acids experiments.

3.2 Experimental design.

After the choice of straw/bagasse ratio and the type of acid to conduct the sequencial assays, an experimental design was performed. Acid concentration (%), time (min) and solids loading (%) were the factor evaluated and the response was the total reducing sugars released in the pretreatment step. Table 2 shows the obtained TRS concentrations under each condition indicated by experimental design.

Table 2: Independent variables associated with their response.

Assay	Acid concentration (%)	Time (min)	Solids loading (%)	TRS ($g.L^{-1}$)
1	0.25	5	10	62.22
2	0.75	5	10	45.30
3	0.25	15	10	37.50
4	0.75	15	10	47.05
5	0.25	5	20	61.12
6	0.75	5	20	45.81
7	0.25	15	20	35.97
8	0.75	15	20	50.22

From Table 2, the range of TRS concentration was 35.97-62.22 $g.L^{-1}$. The highest value was obtained at assay 1, employing 0.25 % of acid concentration, 5 minutes of pretretment and10 % of solids loading. All statistical analyses are performed as following, within 90 % confidence level. Figure 4 shows the observed values versus predicted values.

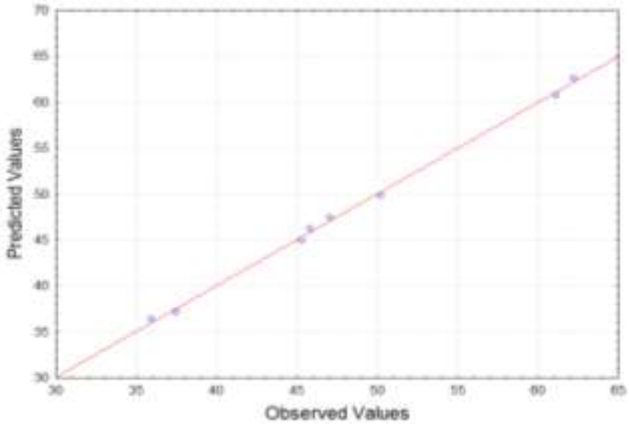

Figure 4. Graphic of normal probability concerning residues.

This is a normal probability graph concerning residues and the observed behavior affirms that the model specifications were achieved, since the experimental points are all close to continuous line. Figure 5 presents the Pareto Chart, that graphically summarizes and display the relative importance concerning a group of data. Effects which the rectangle are to the right of the red line (p=0.1), are statistically significant [19].

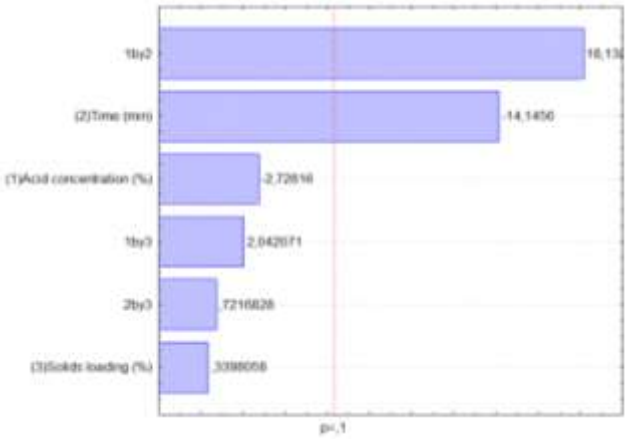

Figure 5. Pareto Chart of TRS to estimate the linear effect of acid concentration (%), time (min), solids loading (%) and the interaction between acid concentration and time, time and solids loading and acid concentration and solids loading (p=0.1).

From Figure 5, the interaction effect between the factors 1 by 2 and the time main effect were significant parameters on influencing the total reducing sugars released during the pretreatment step. Acid concentration and solids loading main effects and interaction effect between the factors 1 by 3 and 2 by 3 were statistically non-significant, within 90 % confidence level. The largest interaction effect was between the factors 1 by 2, acid concentration and time. Furthermore, the factor 2, time, had a negative effect, indicating that its decrease will increase the TRS concentration. Table 3 presents the analysis of variance (ANOVA). Model terms with *p-level* of more than 0.1 were considered non-significant.

Table 3: ANOVA for the linear model of the total reducing sugars optimization.

Factor	Sum of Square	Degrees of freedom	Mean of Square	F value	p-level
R^2=0.99816					
1	8.8831	1	8.8831	7.443	0.2237
2	238.8205	1	238.8205	200.099	0.0449
3	0.1378	1	0.1378	0.116	0.7915
1 by 2	392.4201	1	392.4201	328.794	0.0351
1 by 3	4.9770	1	4.9770	4.170	0.2899
2 by 3	0.6216	1	0.6216	0.521	0.6020
Error	1.1935	1	1.1935		
Total SS	647.0537	7			

As previously showed by Pareto chart, Table 3 shows that p-values higher than 0.1 are associated with non-significant parameters. In addition, test F (that correlates mean of square of the regression and mean of square of the residue) was 10.56-fold higher than tabulated test F. This behavior indicates a significant regression [20].
Figure 6 shows the 3 contour plots of total reducing sugars, for interaction of enzyme dosage and time (a), enzyme dosage and temperature (b) and time and temperature (c).

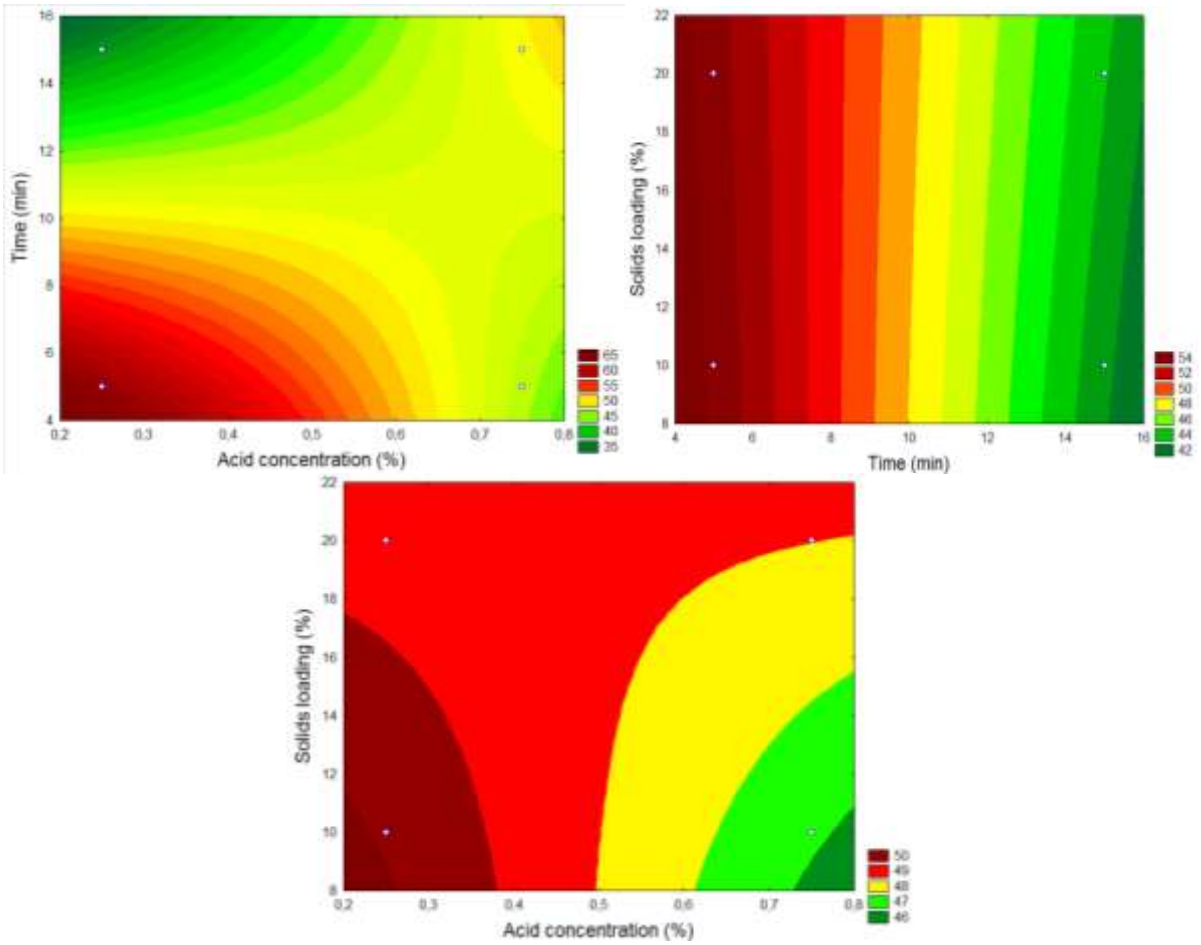

Figure 6. Contour plot of the effect of (a) acid concentration and time, (b) acid concentration and solids loading and (c) time and solids loading on the total reducing sugars releasing during the pretreatment step.

From Figure 6, overall analysis shows that better results, concerning total reducing sugars released throughout the pretreatment step, are obtained when the main factors acid concentration and time are in its lower levels (red region in Figure 6). Solids loading do not presented influence on TRS concentration.

3.3 Enzymatic hydrolysis and fermentation.

Pretreated biomass under condition 1 of the experimental design (Table 2) was submitted to hydrolysis and fermentation. After 72 hours of enzymatic hydrolysis, it was obtained 68.15 $g.L^{-1}$ of total reducing sugars. Hydrolyzed liquor was conducted to fermentation and after 24 hours 25.59 $g.L^{-1}$ of ethanol was reached.

IV. CONCLUSIONS

Concerning dilute inorganic acids evaluated in this work, sulfuric acid outperformed the others. Since it was reached feasible results applying a blend (straw and bagasse), this is a promising way to conduct the pretreatment step from sugarcane wastes. Experimental design pointed the interaction between acid concentration and time the more significant parameter evaluated. It was reached 25.59 $g.L^{-1}$ of bioethanol from a blend of straw and sugarcane bagasse.

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