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Enzymatic Hydrolysis Optimization from Corn Wastes by Experimental Design

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ABSTRACT

An important factor to promote the economic development in a country is correlated with the availability of low-cost energy resources. In this context, corn wastes, such as stover and cob, have been employed as relevant lignocellulosic residues for biofuels generation. Concerning bioethanol production from biomass, enzymatic hydrolysis step is a key point to become the process energy efficient. Taking this into account, it is necessary to perform improvements with regarding to hydrolysis step, aiming the optimization of the global process for second generation ethanol (E2G) production. This work presents a study of enzymatic hydrolysis conditions, using the enzymatic complex Cellic®CTec2, from stover and corn cob (mixed, 1:1 w/w). Biomass was submitted to a pretreatment in sulphuric acid solution (0.25 %, w/w). An experimental design was applied to determinate the optimum conditions for the maximum total reducing sugars (TRS) production, with the variation of enzyme dosage, temperature and time. Acid pretreatment was effective in the removal of hemicellulosic fraction (80.97 %). The highest TRS value from experimental design, 33.5 $g.L^{-1}$, was obtained applying 30 $FPU.g^{-1}_{substrate}$ of enzyme, at 48 hours of hydrolysis, and 50°C.

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

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I. INTRODUCTION

Lignocellulosic biomass has been shown a promising renewable source to increase the sustainable fuels production. In addition, the use of these materials produces biofuels that can be considered carbon-neutral and avoids competition with the food industry [1]. A variety of lignocellulosic substrates has been applied in bioethanol studies around the world. Among them, residues from sugarcane [2,1], rice [3], wheat [4] and corn [5,6] can be highlighted.

Concerning the utilization of corn wastes, stover and cob are in general applied mixed as animal food. Taking this into account, an important way to process both stover and corn cob for second generation ethanol production is investigates the efficiency from a blended feedstock.

Enzymatic hydrolysis is one of the most important steps for E2G production, since the overall process yield is highly dependent on this stage [7]. Experimental designs are powerful tools to promote the optimization of hydrolysis conditions. These tools can evaluate a range of, for example, solids loading, temperature, time and enzyme dosage during the hydrolysis experiments and in this way, choose the best configuration based on the obtained responses [8].

Taking all this into account, this work describes the enzymatic hydrolysis of stover and corn cob, submitted to an acid dilute pretreatment. The studied factors were enzyme dosage, temperature and time of reaction and the evaluated response was the total reducing sugars.

II. MATERIALS AND METHODS

2.1 Raw material.

Stover and corn cob obtained locally to carry out the experiments 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 sulfuric acid pretreatment.

The lignocellulosic corn residues (mixed stover and cob, 1:1 w/w) were submitted to a pretreatment in sulfuric acid solution (0.25 %, w/w), at a solid to liquid ratio of 1:10, in an autoclave at 121 °C for 15 min. 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.

2.3 Chemical characterization.

Both raw and pretreated material (mixed stover and corn cob, at a ratio of 1:1, w/w) was characterized with regard to their chemical composition, according to analytical procedures described by Sluiter *et al*, [9], modified by Rocha *et al*, [10] and validated by Gouveia *et al*, [11].

2.4 Enzymatic hydrolysis.

The enzymatic complex used in the assays was Cellic®CTec2, donated by Novozymes Latin America (Araucária, Paraná, Brazil). The enzymatic activity of the complex was 203 FPU.mL^{-1} [12]. Enzymatic hydrolysis was conducted at solids loading of 10 % (w/v), under batch strategy. The assays occurred with reaction volume of 30 mL, under 150 rpm, in sodium citrate buffer (50 mM and pH 4.8). Enzyme dosage, time and temperature of reaction applied in enzymatic hydrolysis assays were based on experimental design (Statistica®, 7.0 version), as showed in Table 1. After hydrolysis assays, total reducing sugars (TRS) concentrations were quantified by DNS spectrophotometer method [13]. All hydrolysis experiments were performed in triplicate.

Table 1. Independent variables for the experimental design.

Nº. Variable	Level Chosen	
	Low	High
1. Enzyme dosage (FPU.g$^{-1}_{substrate}$)	15	30
2. Time (h)	48	72
3. Temperature (° C)	30	50

III. RESULTS AND DISCUSSION

3.1 Chemical characterization of the raw material.

Two lignocellulosics residues parts (mixed stover and cob) were chemically characterized in terms of cellulose, hemicellulose, lignin, and ash content. The obtained values are presented in Table 2. A comparison with a work proposed in the literature, in which the assays were conducted applying stover and corn cob separately, was performed. Overall, cellulose and hemicellulose were the main components of the biomass.

Table 2: Chemical characterization of *in natura* corn residues (in this work, mixed stover and corn cob 1:1 w/w were evaluated).

Components (%)	This work	Corn stover	Corn cob
		(Santos-Rocha *et al*., 2017b)	
Cellulose	31.3±0.2	36.7±0.2	35.4±0.2
Hemicellulose	32.3±0.1	34.2±0.1	26.9±0.3
Total Lignin	17.4±0.2	14.0±0.1	18.0±0.2
Ashes	1.9±0.1	2.3±0.3	2.4±0.1

As can be seen, there were only slight differences in the chemical composition, when a comparison is performed between the mixed residues and stover and corn cob separately. This trend indicates that these wastes can be applied as a blend, saving costs concerning transport and storage, since both stover and cob are from the same crop. Taking this into account, the chemical characterization is a key point to be considered concerning lignocellulosics conversion into biofuels by the sequential steps of pretreatment, enzymatic hydrolysis, and fermentation.

3.2 Dilute sulfuric acid pretreatment.

The blend of stover and corn cob was pretreated with dilute sulfuric acid and the obtained composition after pretreatment is presented in Table 3. Similarly to *in natura* feedstock, a comparison with the results reported by Santos-Rocha *et al*, [6], in which the assays were conducted applying stover and corn cob separately, was performed.

Table 3: Chemical characterization of pretreated corn residues (in this work, mixed stover and corn cob, 1:1 w/w were evaluated).

Components (%)	This work	Corn stover*	Corn cob*
		(Santos-Rocha *et al*, [6])	
Cellulose	51.8±0.1	49.4±0.1	54.0±0.4
Hemicellulose	9.9±0.1	8.4±0.2	20.9±0.1
Total Lignin	24.4±0.3	20.6±0.2	30.9±0.1
Ashes	1.0±0.1	0.7±0.3	0.7±0.3

*Stover and corn cob were hydrothermally pretreated (1:10 w/w; 195 °C; 10 minutes) (Santos-Rocha *et al*, [6]).

The results presented in Table 3 indicate that the dilute sulfuric acid pretreatment was effective in the hemicellulose removal (80.97 %) and with a low removal of lignin (13.77 %). The high level of hemicellulose removal is a characteristic of dilute acid pretreatment [14,15].

Since removal of hemicelluloses results in significant reduction in cellulase loading required to convert cellulose to glucose and the industrially consolidated fermentation step is conducted by *Saccharomyces cerevisiae* yeast, which ferments more efficiently hexoses, this pretreatment presented a promising way to E2G production [16,17].

Moreover, a comparison between the results conducted in this work with those reported by Santos-Rocha *et al*, [6] emphasizes that a blend from stover and corn cob is a promising way to be industrially applied. This affirmation is supported by the cellulose and hemicellulose contents showed in Table 3, since the blend presented higher cellulose content than corn stover and lower hemicellulose content than corn cob.

3.3 Enzymatic hydrolysis.

Pretreated biomass was submitted to enzymatic hydrolysis by experimental design to evaluate the best condition in terms of total reducing sugars released during the hydrolysis. The results are showed in Table 4.

Table 4: Independent variables associated with their response.

Assay	Enzyme dosage ($FPU.g^{-1}_{substrate}$)	Time (h)	Temperature (°C)	TRS ($g.L^{-1}$)
1	15	48	30	24.1±0.3
2	30	48	30	32.0±0.2
3	15	72	30	22.2±0.1
4	30	72	30	31.3±0.2
5	15	48	50	25.6±0.1
6	30	48	50	33.5±0.1
7	15	72	50	23.2±0.3
8	30	72	50	31.9±0.3

From Table 4, the range of TRS concentration was 22.2-33.5 $g.L^{-1}$. The highest value was obtained at assay 6, employing 30 $FPU.g^{-1}_{substrate}$, 48 hours of hydrolysis under 50 °C. Statistical analyses are performed as following, within 90 % confidence level.

3.4 Experimental design.

Figure 1 shows the normal probability graph. Since experimental points are all close to continuous line, this behavior can sure that the residues are normally distributed [18].

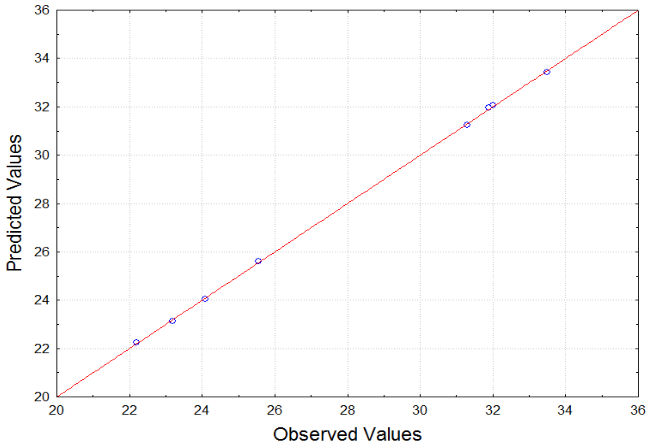

Figure 1. Graphic of normal probability.

Pareto Chart presents quickly and clearly the effects that are statistically significant. Figure 2 shows the Pareto Chart of standardized effects.

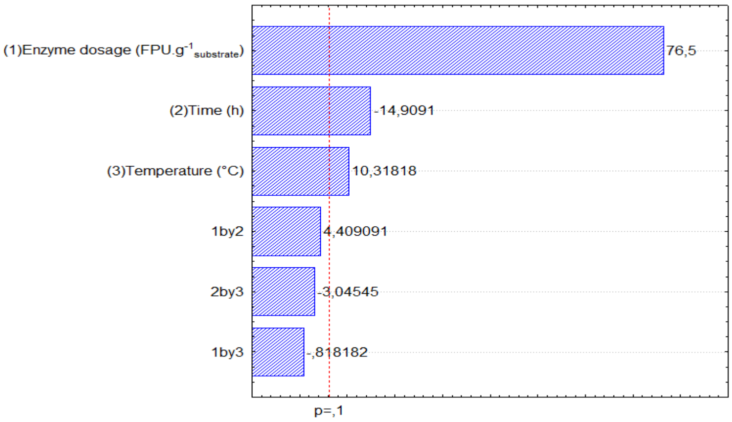

Figure 2. Pareto Chart of TRS to estimate the linear effect of enzyme dosage (FPU.ml^{-1}), time (h), temperature (°C) and the interaction between enzyme dosage and time, time and temperature and enzyme dosage and temperature (p=0.1).

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

Table 5: Analysis of variance (ANOVA) for the linear model.

Factor	Sum of Square	Degrees of freedom	Mean of Square	F value	p-level
R^2=0.99984					
1	141.6245	1	141.6245	5,852.250	0.0083
2	5.3792	1	5.3792	222.281	0.0426
3	2.5765	1	2.5765	106.465	0.0615
1 by 2	0.4705	1	0.4705	19.440	0.1420
1 by 3	0.0162	1	0.0162	0.669	0.5635
2 by 3	0.2245	1	0.2245	9.275	0.2020
Error	0.0242	1	0.0242		
Total SS	150.3156	7			

From the ANOVA test (Table 5), it can be observed that p-values higher than 0.1 are associated with non-significant parameters, as previously showed by Pareto chart. In addition, test F, that correlates mean of square of the regression and mean of square of the residue, was 55.7-fold higher than tabulated test F. This behavior indicates a highly significant regression [19].

The 3 graphs of total reducing sugars, for interaction of enzyme dosage and time (a), enzyme dosage and temperature (b) and time and temperature (c), called contour plot, are shown in Figure 3.

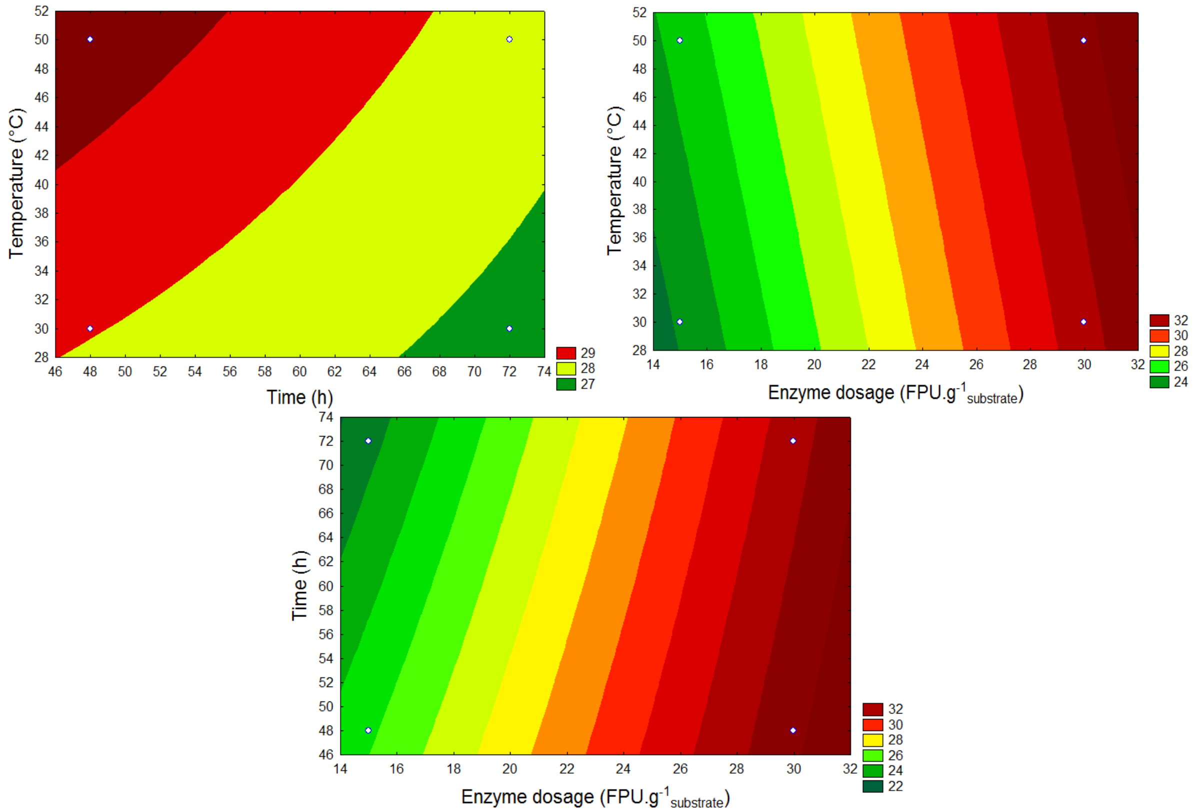

Figure 3. Contour plot of the effect of (a) enzyme dosage and time, (b) enzyme dosage and temperature and (c) time and temperature on the total reducing sugars releasing during the enzymatic hydrolysis.

It can be observed from Figure 3, that there is a positive influence from the enzyme dosage and temperature on the evaluated response, since an increase on total reducing sugars concentration occurs when these factors are increased (red region in Figure 3). On the other hand, concerning the time effect, opposite behavior is showed. Overall analysis shows that better results are obtained as enzyme dosage and temperature increase and as time is reduced throughout the enzymatic hydrolysis assays.

IV. CONCLUSIONS

The results obtained in this work and their evaluation by experimental design reach the conclusion that maximum sugars liberation occurs with 30 FPU.mL^{-1} of enzyme dosage and 48 h of enzymatic hydrolysis, conducted under 50 °C. In addition, only main effects were significant on influencing the TRS concentration, released throughout the enzymatic hydrolysis. The study can affirms that the application of a blend of stover and corn cob for second generation ethanol production can be a promising way to conduct these residues for a high add-value activity.

REFERENCES

[1] M.S.R. Santos-Rocha, B. Pratto, R. Sousa Jr, R.M.R.G. Almeida, and A.J.G. Cruz, A kinetic model for hydrothermal pretreatment of sugarcane straw, *Bioresource Technology*, 228, 2017a, 176-185.

[2] F.I.B. Ogando, J.A.S. Sartori, N.T.R. Magri, and C.L. Aguiar, Pretreatment of sugarcane top leaves by ozonation as a promotion of susceptibility to hydrolysis, *Journal of Bioenergy and Food Science*, 3(4), 2016, 197-206, 2016.

[3] E. Cabrera, M.J. Munõz, R. Artín, I. Caro, C. Curbelo, and A.B. Díaz, Alkaline and alkaline peroxide pretreatments at mild temperature to enhance enzymatic hydrolysis of rice hulls and straw, *Bioresource Technology*, 167, 2014, 1-7.

[4] M-R. Zoulikha, M. Thierry, Z.J-M. Qiuyu, A. Nouviaire, and R. Sid-Ahmed, Combined steam-explosion toward vacuum and dilute-acid spraying of wheat straw. Impact of severity factor on enzymatic hydrolysis. *Renewable Energy*, 78, 2015, 516-526.

[5] M.S.R. Santos-Rocha, M.C.S. Silva, W.R.O. Pimentel, and R.M.R.G. Almeida, Acid pretreatment of corn stover for production of second generation ethanol, *Engevista*, 18(2), 2016, 412-423.

[6] M.S.R. Santos-Rocha, R.A.B. Souza, G.M. Silva, A.J.G. Cruz, and R.M.R.G. Almeida, Hydrothermal pretreatment of corn residues for second generation ethanol production, *Scientia Plena*, 13(3), 2017b, 1-13.

[7] L.N. Siqueira, E.A. Guarda, P.M. Guarda, R.B.R. Silva, and R.S. Barbosa, Yield of hydrolysis and production of lignocellulosic ethanol from elephant grass biomas, *Journal of Bioenergy and Food Science*, 3(4), 2016, 191-196.

[8] M.D.M. Samsudin, M.M. Don, N. Ibrahim, R.M. Kasmani, Z. Zakaria, and K.S. Kamarudin, Batch fermentation of bioethanol from the residues of Elaeis Guineensis: optimisation using response surface methodology, *Chemical Engineering Transactions*, 56, 2017, 1579-1584.

[9] Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker, *Determination of structural carbohydrates and lignin in biomass,* (Technical Report NREL/TP-510-42618, 2008).

[10] G.J.M. Rocha, F.T. Silva, A.A.S. Curvelo, and G.T. Araújo, A fast and accurate method for determination of cellulose and polyoses by HPLC. *In: Fifth Brazilian symposium on the chemistry of lignins and other wood components*, 1997, 1-8.

[11] E.R. Gouveia, R.T. Nascimento, and A.M. Souto-MAIOR, Validação de metodologia para a caracterização química de bagaço de cana-de-açúcar, *Química Nova*, 32(6), 2009, 1500-1503.

[12] T.K. Ghose, Measurement of cellulase activities, *Pure and Applied Chemistry*, 59(2), 1987, 257–268.

[13] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry*, 31, 1959, 426-428.

[14] C-S. Lau, G.J. Thoma, E.C. Clausen, and D.J.Carrier, Kinetic modeling of xylose oligomer degradation during pretreatment in dilute acid or in water, *Industrial & Engineering Chemistry Research*, 53, 2014, 2219-2228.

[15] S.C. Pereira, L. Maehara, C.M.M. Machado, and C.S. Farinas, Physical-chemical-morphological characterization of the whole sugarcane lignocellulosic biomass used for 2G ethanol production by spectroscopy and microscopy techniques, *Renewable Energy*, 87, 2016, 607-617.

[16] L. Viikari, J. Vehmaanperä, and A. Koivula, Lignocellulosic ethanol: from science to industry. *Biomass &Bioenergy*, 46, 2012, 13-24.

[17] O.O. Kuloyo, J.C. Preez, M.P. García-Aparicio, S.G. Kilian, L. Steyn, and J. Görgens, Opuntia ficus-indica cladodes as feedstock for ethanol production by Kluyveromyces marxianus and Saccharomyces cerevisiae, *World Journal of Microbiology and Biotechnology*, 30, 2014, 3173-3183.

[18] Calado, and D.C. Montgomery, *Planejamento de experimentos usando o Statistica* (Rio de Janeiro: E-papers, 2003).

[19] B. Barros-Neto, I.S. Scarminio, and R.E. Bruns, *Como fazer experimentos. Pesquisa e desenvolvimento na ciência e na indústria* (Campinas: Editora da Unicamp, 2001).

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