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I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL

XXIV SINAFERM • XV SHEB • XV ENZITEC • FLORIANÓPOLIS • 2024

Creating connections between biotechnology and industrial sustainability

August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil

BIOPROCESS ENGINEERING

OPTIMIZATION DRIVEN BY EXPERIMENTAL DESIGN OF ALKALINE GLYCEROL ORGANOSOLV PRETREATMENT AT LOW TEMPERATURE FOR ENHANCED SACCHARIFICATION OF TOBACCO STEM

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ABSTRACT

This study investigated the potential of tobacco stem as a raw material for the production of chemical intermediates through alkaline glycerol organosolv (al-GO) pretreatment at low temperature (120 °C) followed by enzymatic hydrolysis. Using an experimental design, the effects of solid loading (6.6 to 23.4% (w/v)), glycerol (0 to 100% (v/v)) and NaOH (0 to 6.4% (w/v)) were evaluated for lignin removal, polysaccharide retention and enzymatic hydrolysis efficiency. The optimized condition included solid load of 15% (w/v), NaOH load of 3% (w/v) and glycerol load of 50% (v/v), allowing lignin removal of 58.1%, polysaccharides yield of 55.9%, 54.4 g/L of total reducing sugars and 75.2% enzymatic hydrolysis yield.

Keywords: Polysaccharides Recovery. Lignin Removal. Enzymatic Hydrolysis. Lignocellulose. Experimental Design

1 INTRODUCTION

Tobacco is one of the world's primary non-edible agricultural crops.[1] Despite its economic significance, the tobacco agroindustry has severe environmental impacts, such as the release of 84 million tons of CO_2 annually and the generation of substantial amounts of waste, including the stem and the stalk of the plant, which are not utilized in the cigarette production chain.[2] The integration of these residues into biorefinery processes offers a promising avenue to add value to them and produce compounds of high commercial interest. The recalcitrant lignocellulosic structure requires pretreatment strategies for complete use of biomass. Among these, glycerol organosolv pretreatment is promising for enhancing enzymatic digestibility, owing to its high delignification capacity and polysaccharides retention.[3] When pure glycerol is used in pretreatment, high temperatures (> 200 °C) are typically required, significantly increasing energy costs.[4] Thus, in organosolv pretreatments, an alternative is the use of alkaline catalysts such as NaOH, reducing the temperature needed.[4] However, there are no studies in the literature that simultaneously optimize solid load, glycerol load and NaOH load in alkaline glycerol organosolv (al-GO) pretreatment at low temperature. This work evaluates for the first time the effects of al-GO pretreatment on tobacco stem. Through a central composite rotatable design (CCRD), for the first time the parameters of al-GO pretreatment (solid load, NaOH and glycerol) at low temperature (120 °C) were assessed for lignin removal, polysaccharides preservation and enzymatic hydrolysis efficiency. The optimized conditions that maximize the response variables were calculated using a desirability function.[5]

MATERIAL & METHODS

Initially, the tobacco stems were milled in a knife mill (TE-631/2) and subsequently sieved within the range of 30 to 40 mesh using a vibrating sieve system (Model No. SERIES 6273, BERTEL, Brazil). Following this, the biomass underwent extractive fraction removal using a solution of 8:2 ethanol/water, at a solid loading of 5% (w/v). The mixture was agitated at 2000 rpm and at 60 °C for 1 h. The solids derived from this stage were separated for subsequent pretreatment procedures. The al-GO pretreatment was conducted in 500 ml Erlenmeyer flasks containing the reaction medium with solid loading and reagent concentrations corresponding to each experimental condition. These flasks were placed within an autoclave (Phoenix Luferco AV-100), where they underwent reaction at a constant temperature of 120 °C, for 1 h, counted after reaching the desired temperature. Upon completion of reaction time, the heating was ceased, and the system was cooled to room temperature. The liquid and solid fractions were separated, with the solid fraction being washed with tap water until neutral pH was reached. To ensure neutrality, pH of aliquots of the washing water was determined using a pH meter. Subsequently, the solids were dried in a forced air circulation oven until constant weight and characterized in terms of cellulose, hemicellulose, lignin, extractives, and ash content according to the Analytical Laboratory Protocol of the National Renewable Energy Laboratory (NREL).[6]

The experiments followed a 2^3 central composite rotatable design (CCRD), covering 6 tests in axial conditions and 3 replicates at the central point, totaling 17 runs. The independent variables comprised solid loading (6.6, 10, 15, 20, and 23.4% (w/v)), NaOH loading (0, 1, 3, 5, and 6.4% (w/v)), and glycerol loading (0, 20, 50, 80, 100% (v/v)). Lignin removal, polysaccharide yield, enzymatic hydrolysis efficiency and total reducing sugars obtained after saccharification were the response variables. Multiple regression was applied to the experimental data, to obtain second-order polynomial models capable of predicting the conditions that maximize the response variables. The statistical significance of the effects was tested by ANOVA, and the non-significant effects ($p > 0.05$) were removed from the models. The adequacy of the models were evaluated using the determination coefficient

(R2), model p – value and lack of fit p – value. Equation (1) presents the generalized second-order polynomial equation used to fit the experimental data.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} X_i X_j \tag{1}$$

In Equation (1), β_0, β_i, β_{ii}, β_{ij}, are the regression coefficients for terms of interception, linear, quadratic and interaction, respectively. X_i and X_j are the independent variables. Once the models were obtained and the statistical adequacy tested, it was possible to generate response surfaces to visually analyze the behavior of the process. To maximize response variables, a multi-response optimization was performed using a desirability function.[5] Statistical analysis of the results was performed using Statistica software v. 13.5 (TIBCO Software Inc., Palo Alto, CA, USA). Enzymatic hydrolysis of all solid fractions was carried out to evaluate the influence of each pretreatment condition on enzymatic digestibility. Reactions were conducted in 250 ml Erlenmeyer flasks utilizing a solid loading of 10% (w/v) in 50 mM sodium citrate buffer (pH 4.8). The enzyme loading (Cellic ®Ctec2) was 12 FPU/g$_{biomass}$ (Filter Paper Unit per gram of biomass).[7] Flasks were incubated in an orbital shaking incubator (TECNAL TE-424) at 150 rpm and 50 °C for 72 h.[7] Glucose, celobiose and xylose concentrations after saccharification were determined via High-Performance Liquid Chromatography (HPLC). Assays were performed in duplicate.

2 RESULTS & DISCUSSION

From the data of lignin removal and polysaccharide yield (Table 1), it was possible to obtain statistically valid and predictive linear regression models for these response variables. For both regressions, the lack of fit was not significant (p - value (lack of fit) > 0.05), the models were significant (p - value (model) < 0.05) and explained up to 99.77% of the variance for polysaccharide yield and up to 95.21% for lignin removal (Table 2). The NaOH load was the independent variable that most influenced the response variables, since it presented the highest regression coefficients. For polysaccharide yield, its impact was negative, while for lignin removal it was positive, indicating that NaOH was the most responsible for depolymerizing the lignocellulosic network. Presenting the opposite effect, glycerol had a positive coefficient for polysaccharide yield and negative for lignin removal. This indicates that glycerol, at low temperature, protects polysaccharides, but hinders the removal of lignin. Solid load had a positive coefficient for polysaccharide yield and negative for lignin removal, being an obstacle for depolymerization of lignin and polysaccharides. This was due to mass transfer limitations and worse medium homogenization imposed by the increase of solid load.[8]

Table 1. Biomass composition, pretreatment effects and enzymatic hydrolysis results after each run.

	Experimental design			Biomass composition (%)			Pretreatment Effects (%)		Enzymatic Hydrolysis - 72 h	
Run	Solid load (% w/v)	NaOH (% w/v)	Glycerol (% v/v)	Cellulose	Hemicellulose	Lignin	Polysaccharide Yield	Lignin Removal	Enzymatic Yield (%)	Total Reducing Sugars (g/L)
	Control (*in natura*)			27.67±0.58	21.58±0.21	33.51±1.42	-	-	-	-
1	10	1	20	39.28±2.68	18.08±0.11	38.56±3.58	69.69	31.14	50.12	33.39
2	20	1	20	34.69±1.66	17.94±0.52	39.75±0.89	76.62	14.94	40.56	24.74
3	10	5	20	51.07±3.52	18.62±0.26	28.03±2.09	54.37	67.85	50.09	40.87
4	20	5	20	45.44±1.11	18.23±0.18	32.71±0.30	57.62	56.49	71.75	53.01
5	10	1	80	41.09±0.60	18.10±0.45	31.76±0.94	76.29	39.83	56.79	38.91
6	20	1	80	35.59±1.73	18.93±0.47	38.75±1.06	85.18	11.03	36.26	22.93
7	10	5	80	42.73±0.39	19.09±0.06	35.71±3.10	53.7	54.41	80.21	57.34
8	20	5	80	42.22±0.73	18.24±0.08	33.31±0.68	55.97	54.68	72.33	50.71
9	6.6	3	50	47.02±0.40	20.09±0.25	28.98±1.07	56.57	64.09	57.68	45.03
10	23.4	3	50	38.53±0.07	18.88±0.96	35.62±2.47	65.36	40.39	71.80	47.86
11	15	0	50	31.85±0.10	18.40±0.05	35.56±0.31	88.01	8.46	22.17	12.88
12	15	6.4	50	47.37±2.21	20.33±0.26	29.52±1.10	56.34	63.9	72.45	56.98
13	15	3	0	41.61±0.40	20.70±0.01	33.75±0.38	55.81	55.57	67.75	48.94
14	15	3	100	37.24±0.58	18.32±0.17	40.47±0.19	60.6	35.12	67.75	43.72
15	15	3	50	39.55±2.52	21.39±0.79	32.80±0.45	56.15	55.59	76.63	54.22
16	15	3	50	41.32±1.13	21.78±0.43	31.10±0.17	55.63	59.7	73.55	53.85
17	15	3	50	40.97±0.76	22.14±0.92	31.49±0.54	55.99	58.94	75.32	55.24

It was not possible to obtain statistically valid and predictive models for the response variables of enzymatic hydrolysis. By individual analysis of the results, al-Go pretreatment at low temperature was able to reduce the recalcitrance of biomass and achieve enzymatic hydrolysis yields above 70% (runs 4,7,8,10,12,15,16 and 17) and concentration of reducing sugars above 50 g/L (runs 4,7,8,12,15,16,17)(Table 1). Al-GO pretreatment was applied in sugarcane bagasse in conditions close to run 9 of this work (120 C, 1 h, 5% Solid load, 50% (w/w) glycerol and 9% (w/w) ammonia), achieving lower lignin removal (49.34%) and higher recovery of polysaccharides (92.58% cellulose and 84.60% hemicellulose) in relation to this study.[9] These differences are probably due to different catalytic strenghts of NaOH and ammonia. Comparing the enzymatic hydrolysis data after these pretreatment conditions, 6.86 g/L of total reducing sugars were obtained from sugarcane bagasse, against 45.03 g/L in this study.[9]

The optimization indicated that the condition that maximized lignin removal and polysaccharide yield was the central point of the experimental design (15% solid load, 3% NaOH, 50% glycerol). This condition presented average lignin removal of 58.1% and polysaccharide yield of 55.9%. In addition, the central point allowed the average production of 54.4 g/L of total reducing sugars and an average enzymatic hydrolysis yield of 75.2%.

Table 2. Regression coefficients and statistical parameters of each independent variable factor, solid load (X_1), NaOH (X_2) and glycerol (X_3).

| Response Variables	Factors	Regression Coefficients	Standard Error	t - value	p - value
Polysaccharide Yield (% w/w) $R^2 = 0.9977$ p – value (lack of fit) = 0.0871 p – value (model) < 0.0001	Constant	55.87	0.44	126.72	0.0000
	X_1	2.65	0.21	12.70	0.0000
	X_1^2	1.98	0.23	8.70	0.0000
	X_2	-10.98	0.21	-51.33	0.0000
	X_2^2	6.96	0.25	28.38	0.0000
	X_3	1.54	0.21	7.35	0.0001
	X_3^2	1.02	0.23	4.43	0.0022
	X_1X_2	-1.29	0.27	-4.73	0.0015
	X_2X_3	-2.19	0.27	-8.03	0.0000
Lignin Removal (% w/w) $R^2 = 0.9521$ p – value (lack of fit) = 0.1282 p – value (model) < 0.0001	Constant	55.20	2.32	23.77	0.0000
	X_1	-7.03	1.44	-4.87	0.0006
	X_2	17.86	1.48	12.08	0.0000
	X_2^2	-8.67	1.63	-5.31	0.0003
	X_3	-3.29	1.45	-2.27	0.0464
	X_3^2	-4.12	1.53	-2.69	0.0227
	X_1X_2	4.24	1.88	2.25	0.0481
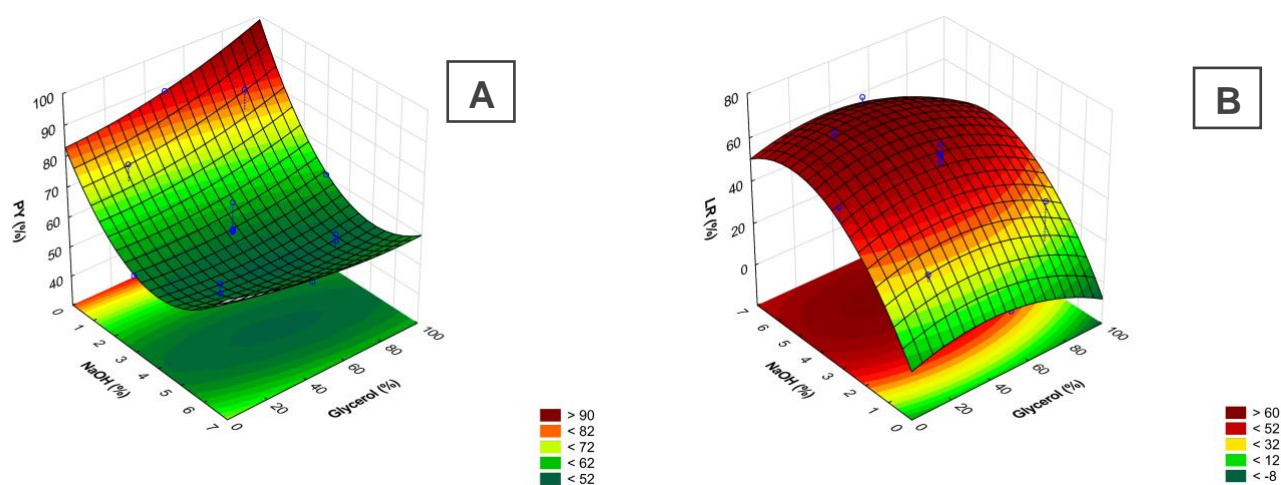
Figure 1 Response surfaces generated by CCRD, with fixed solids loading at 15%. (A); Polysaccharide Yield (PY) (%) as a function of NaOH (%) and glycerol (%). (B); Lignin Removal (LR)(%) as a function of NaOH (%) and glycerol (%).

3 CONCLUSION

This work highlights for the first time the feasibility of reducing recalcitrance and achieving high yields and concentrations of reducing sugars after enzymatic hydrolysis using pre-treatment al-GO in tobacco stems at low temperature. This approach can be an alternative for valuing tobacco residues and integrating them into biorefinery platforms.

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ACKNOWLEDGEMENTS