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Bioethanol Production from Residual Tobacco Stalks

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RCSS, LAA and VAC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LAA, MSS and LFFS managed the hydrolysis and analyses of the study. Author MSAM managed the HPLC analysis. Authors RCSS and LAA managed the literature searches. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: In this work, bioethanol production from residual tobacco stalks was investigated.
Place and Duration of Study: Stalks sampling was in South Brazil crops and experiments in chemistry laboratories at Unisc, after tobacco leaf harvest.
Methodology: Pretreatments were conducted with sulfuric acid solution (1 to 3%) in an autoclave (121℃) for 30 to 90 min. Enzymatic hydrolysis was perf ormed with two enzymes, CTec2 and HTec2 (Novozymes). Fermentation (*Saccharomyces cerevisiae*) was conducted with hydrolysate obtained in selected conditions of acid pretreatment and enzymatic hydrolysis.
Results: In enzymatic hydrolysis, 38.1% glucose was obtained from the pretreated solid. The ethanol yield was 0.06 to 0.19 g of ethanol per g of dried and milled tobacco stalk. The results showed that it is possible to use this biomass for the ethanol production; however, some saccharification variables can still be modified to increase the conversion.

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Conclusion: Thus, tobacco stalk, which has no economic value, appears to be a source of monosaccharides for the fermentation and production of bioethanol. Furthermore, the exploitation of tobacco stalks may be of great importance to agriculture and industry as currently over 300,000 ha of land in Southern Brazil is used for tobacco production, and after harvesting, the stalks simply remain unused in the soil.

Keywords: Enzymatic hydrolysis; ethanol; fermentation; tobacco stalks.

1. INTRODUCTION

Excessive consumption of fossil fuels has resulted in the generation of high levels of pollutants. Annual fossil fuel production is projected to decrease in upcoming decades. In this scenario, renewable fuels have emerged as a new alternative [1].

In Brazil, ethanol production was established from sugarcane, and currently, viable ethanol production exists from sugarcane bagasse at the industrial level [2].

Technological developments have simultaneously reduced the environmental impact and price of bioethanol production. According to Siqueira et al. [3], efforts have been focused on developing more efficient fermentative organisms, cheaper fermentation substrates and optimized fermentation conditions.

Brazil has sufficient land space to significantly increase both food and bioethanol production; in Goiás State alone, the planted sugarcane area has increased almost six-fold (142 mil ha to 847 mil ha) over ten years [4]. Moreover, lignocellulosic materials are important for agroindustrial diversification and waste recovery in the biofuel chain.

Ethanol production from lignocellulosic material consists essentially of biomass chemical/enzymatic hydrolysis for its saccharification, followed by attack by microorganism capable of fermenting monosaccharides, and finally, ethanol production [5,6].

In this context, most plant residues with a high concentrations of cellulose could be a substrate for bioethanol production.

According to Martín et al. [7] ethanol was produced at a higher rate from tobacco stalks, 0.38-0.39 g/g of initial fermentable sugars. Shen et al. [8] also worked with improvements of bioethanol production from tobacco stalks. They reached 62.4% of removal of hemicellulose and lignin when used hydrogen peroxide in the pretreatment before enzymatic hydrolysis of cellulose of the tobacco stalks. Therefore, the potentiality of ethanol production from tobacco biomass pretreated can be higher. Veramendi et al. [9] and Farran et al. [10] obtained leaf and stalks hydrolysates from transgenic tobacco plants and reached an ethanol average of 20–40% higher than normal tobacco.

Besides that, Wang et al. [11] carried out the hydrolysis and fermentation of liginin in the tobacco residues with metals (Mn, Fe and Zn) as catalysts (metal-enzyme) obtaining 91.2 $mg \cdot g^{-1}$ ethanol.

Upon analyzing tobacco production, great potential was observed for the production of bioethanol because this crop uses approximately 323,700 hectares of agricultural area (2013/2014) in addition to 731,390 tons of tobacco leaves produced for the cigarette industry. It is noteworthy that 98 % of tobacco crops are concentrated in Southern Brazil in the states of Rio Grande do Sul, Santa Catarina and Paraná, totaling 652 cities and 162,000 producers [12].

The harvesting stages are common among producers in southern Brazil. The harvest of tobacco leaves occurs gradually, starting with the lower leaves and concluding with the upper ones. Thus, after harvesting, all that remains of the tobacco plant are the stalks, which are rich in cellulose. Currently, this material is cut and left in the field and does not provide any functional purpose for the soil or the next crop. Therefore, the use of these stalks provides an interesting opportunity to explore that may benefit both agriculture and industry.

The purpose of this work is to produce bioethanol from tobacco stalks using a pretreatment with dilute sulfuric acid, enzymatic hydrolysis and

fermentation with *Saccharomyces cerevisiae*. This optimized process may provide an alternative for waste management because tobacco stalks are rich in fiber and have no economic value, even though they are highly produced in several countries that currently grow tobacco leaves.

2. MATERIALS AND METHODS

The pretreatment process and biomass hydrolysis were defined according to previous studies regarding lignocellulosic raw materials [13,14] and tobacco stalks [15,16].

The tobacco stalks were collected after the tobacco leaves were harvested for cigarette production at the experimental station of the Association of Tobacco Growers of Brazil (AFUBRA) in Rio Pardo - RS, Brazil (Fig. 1). The samples were dried in an oven at 50℃ and comminuted with 20 - 80 mesh sieves (0.841 mm - 0.177 mm particle size). The fraction retained on the 80 mesh sieve was retained for sugar compositional analysis, which was carried out as described in Sluiter et al. [17]. For pretreatment and enzymatic hydrolysis, the sample was comminuted to < 1.00 mm particle size. The composition of polysaccharides was determined by Sluiter et al. [18].

2.1 Acid Pretreatment

The pretreatment conditions with sulfuric acid were performed with 10% (w/v) biomass. The acid concentration ranged from 1 to 3%, and the pretreatment time ranged from 30 to 90 min. All experiments were performed in triplicate, in an experimental design 2^2 plus central point.

All material was pre-soaked at room temperature for some minutes in sulfuric acid solution to ensure thorough wetting Pretreatment was performed in an autoclave at 121℃ using a borosilicate glass container resistant to high temperatures. After pretreatment the insoluble fractions (solids) were recovered by filtration and washed to remove residual acid until pH 5.0 was achieved. The hydrolysates were filtered, their sugar composition was analyzed, and their solid phase was dried and weighed.

The efficiency of the acid pretreatment was calculated in relation to the remaining solid content after acid hydrolysis [19] and in relation to the hexose and pentose content of the hydrolysate by equation 1, where $mass_i$= initial stalks biomass and $mass_f$= biomass after pretreatment.

$$Eficiency(\%) = \frac{mass_i - \mathrm{mass}_f}{\mathrm{mass}_i} * 100$$

Fig. 1. Tobacco plant before and after leaves harvesting

2.2 Enzymatic Hydrolysis

New enzymes were used in the hydrolysis, namely, Cellic CTec2 (VCNI0013) and Cellic HTEC2 (VHN00003) from Novozymes, both which were derived from *Trichoderma reesei* strains. A portion of the sample (0.5 g) was hydrolyzed in 10 mL of citrate buffer solution (pH 5) containing 40 FPU of enzyme. The test for each hydrolysis condition was performed in triplicate.

Hydrolysis was performed in an Incubator Shaker MA420 (Marconi brand) at 50℃ and 300 rpm for 74 h. Sample aliquots of 0.5 mL were collected at 0, 24, 43, 60 and 74 h for sugar analysis.

2.3 Fermentation

After biomass saccharification, the hydrolysate was centrifuged, filtered and fermented with *Saccharomyces cerevisiae* for alcohol production.

For each 50 mL of hydrolysate, 0.3 g of monopotassium phosphate, 0.15 g of magnesium sulfate and 0.75 g of yeast extract was added. This mixture was autoclaved at 121 ℃ for 30 min. After cooling, 0.75 g of yeast was added (Safale S-04) to the sterile hydrolysate. This mixture was incubated in an incubator Shaker MA420 (Marconi brand) at 30℃ and 100 rpm for up to 28 h.

The determination of ethanol was performed by gas chromatography coupled to a flame ionization detector and autosampler Headspace (HS / GC-FID).

2.4 Determination of Total Reducing Sugars

The total reducing sugars was determined by a reaction with DNS solution (2 g of 3,5-dinitrosalicylic acid and 60 g of potassium sodium tartrate - Rochelle salt diluted in 80 mL of 0.5 mol sodium hydroxide L^{-1}). The acid hydrolysates were initially neutralized with NaOH solution.

The reaction with DNS was conducted by heating to boiling for 5 min, cooling to room temperature and then filtering. The product was analyzed at 540 nm in a spectrophotometer UV/ visible (V-1200, Pró-Análise) using glucose for construction of the calibration curve [20].

2.5 Determination of Pentoses and Hexoses

High performance liquid chromatography (HPLC, Shimadzu, Japan) with a diode array detector (DAD, SPD-M20A) was used for sugar analysis. For this purpose, samples were derivatized with p-aminobenzoic acid (PABA) [21] and diluted in mobile phase B as described below.

Chromatographic analysis was carried out in a Micropac RP-C_{18} column at 25℃ with two mobile phases. Mobile phase A was prepared from 20 mmol $TBAHSO_4$ L^{-1} in phosphate buffer solution (0.1 mol L^{-1}, pH 6.5) and methanol (50:50 (v/v)). Mobile phase B was prepared with 20 mmol $TBHSO_4$ L^{-1} in phosphate buffer solution (0.05 mol L^{-1}, pH 6.5). Orthophosphoric acid was used for pH adjustment. The gradient elution of the mobile phase was as follows: 100% of A for the first 20 min, 25% of B and 75% of A for the next 7 min and 100% of A until 30 min. The injected volume for each sample was 10 µL, and the total flow of the mobile phase was 0.8 mL min^{-1}. The DAD detector was set to a scanning range of 210 - 400 nm and the derivatized sugar was quantified at 303 nm. The data were collected through LC Solution software (Shimadzu) and further processed in OriginPro 8.5 software.

The analytical curves were plotted for hexoses with glucose and for pentoses with xylose and arabinose, and both compounds were at a concentration of 0.5 to 10 mg mL^{-1}.

2.6 Ethanol Determination

Determination of the ethanol concentration during the fermentation was performed by gas chromatography HS/GC-FID using a ZB5 column (60 m x 0.25 mm x 0.25 µm) at 60℃ to 100℃ (5℃ min^{-1}) coupled to a flame ionization detector at 270℃. The headspace injection conditions were 85ºC of furnace heating by 10 min with shaking every 0.5 min. The injection was accomplished at 100℃ in the injector port in splitless mode. The analytical curve was determined with aqueous solutions prepared from absolute ethanol in the same conditions as the hydrolyzed sample.

The ethanol content was determined in the fermentation extract based on the initial dry biomass for the calculation step.

2.7 Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA). Statistical analysis revealed a significant interaction between concentrations and time ($P < 0.05$). The differences were considered significant when $P < 0.05$. Graphpad Prism 6.0 software was used.

3. RESULTS AND DISCUSSION

3.1 Acid Pretreatment

The tobacco stalk samples were subjected to five different pretreatment conditions in triplicate. Pretreatment optimization resulted in a greater concentration of pentoses than hexoses, as expected. The data in Table 1 show that reaction conditions 1, 2 and 5 have greater hydrolysis efficiency than the other conditions, as evidenced by the relationship between sugars in the hydrolysate.

According to Avci et al. [22] and Dagnino et al. [23], formation-inhibiting compounds from fermentation and enzymatic hydrolysis are favored when the acid concentration and duration of pretreatment are increased. It is essential to define not only the conditions to release fermentable sugars but also those that produce minimum inhibitory compounds, such as furfural and hydroxymethyl furfural. These inhibitors were not observed in the chromatograms of the hydrolysates. Thus, it is assumed that if they were produced, they were produced at low concentrations.

Based on the results of the pentose and hexose content shown in Table 1, it was observed that the best hydrolysis condition was performed with 3 % sulfuric acid solution for 90 min. In this condition, it was possible to have a greater amount of pentoses in relation to hexoses, according to the pentose/hexose ratio results.

Efficiency analysis of the pretreatment also considered the reduction of solid mass (Table 2). Likewise, there was a higher formation of sugars in the hydrolysate, illustrating greater pretreatment efficiency. According to the results obtained for the sugars in the hydrolysate, the removal of hemicellulose was greater than that of cellulose. These results showed that acid pretreatment has efficiency over the biomass without a significant difference ($P>0.05$) between the final solid masses after different acid attacks. However, in pretreatment, glucose polysaccharides were lost in the acid hydrolysate before enzymatic action.

3.2 Enzymatic Hydrolysis

The 30-min pretreatment did not show positive results for hemicellulose removal from the biomass of tobacco stalks; therefore, the enzymatic hydrolysis experiments were performed with solid samples from the following pretreatments at 121℃: 90 min with 1 and 3 % H_2SO_4 and 60 min with 2% H_2SO_4. Enzymatic hydrolyses were conducted with the three selected pretreatments in addition to the two enzymes (HTec2 and Ctec2). The results regarding the production of total reducing sugars from enzymatic hydrolysis are shown in Table 3.

The enzyme CTec2 showed better performance than HTec2 in the same pretreatment conditions. With Ctec2, for conditions 1 (3% H_2SO_4/90 min), 2 (1% H_2SO_4/90 min) and 5 (2% H_2SO_4/60 min), the concentrations of total reducing sugars were 53, 38 and 6%, respectively, which were higher than those with HTec2.

Compared to CTec2, the HTec2 enzyme is more specific to xylose because it contains endoxylanase. According to the results obtained for pretreatment, pentoses, such as xylose, were the most removed sugars in the acid hydrolysate. Furthermore, the CTec2 enzyme is a cellulase with β-glucosidase activity, which favors cellobiose hydrolysis.

Table 1. Composition of the hydrolysates obtained in the pretreatment of tobacco stalk biomass

Sample	Acid concentration (%)	Time (min)	Total reducing sugars	Hexoses[a] (mg mL^{-1})	Pentoses[b] (mg mL^{-1})	Ratio Pentoses/ hexoses
1	3	90	20.3 ± 0.8	2.0	9.9	5.0
2	1	90	17.7 ± 0.7	2.0	8.1	4.1
3	3	30	13.3 ± 0.8	2.1	6.9	3.3
4	1	30	8.6 ±0.1	1.3	3.1	2.4
5	2	60	16.6[c]	1.0	4.5	4.5

a) Data in glucose content; b) Data in xylose + arabinose content; c) Only one result

Table 2. Efficiency of the acid pretreatment of tobacco stalks

Sample	Acid concentration (%)	Time (min)	Eficiency (%)
1	3	90	54,2
2	1	90	51,3
3	3	30	48,6
4	1	30	41,2
5	2	60	52,5

Table 3. Results of total reducing sugars during enzymatic hydrolysis

Enzymes	Time (h)	Total Reducing sugars (mg mL^{-1})		
		#1	#2	#5
HTEC2	1	2,1	2,1	3,2
	24	3,7	4,7	7,1
	43	5,0	4,9	7,5
	60	4,8	4,4	8,7
	74	4,6	5,5	9,3
CTEC2	1	3,0	3,0	3,2
	24	5,4	5,9	7,7
	43	6,4	6,6	8,7
	60	7,0	7,7	9,1
	74	7,0	7,7	9,8

#1, #2, #3 – Conditions of the pre-treatments shown in Table 1 which the solid phases were selected for the enzymatic hydrolysis

Analyzing only the CTec2 enzyme, which had the highest production of total reducing sugars, it was observed that pretreatment condition 5 (60 min/ 2% H_2SO_4) had the best results for enzymatic hydrolysis.

Results regarding enzymatic hydrolysis with the same enzymes but for hydrothermally pretreated sunflower stalks were obtained by Jung et al. [24]. These results were 10% higher than the result of this study, to tobacco stalks, and, contrary to what we found, they were better for CTec2. The hydrolysis depends on several factors such as cellulose crystallinity, different treatments and, during thermal-chemical pretreatment, the fibers first side group to react, in this case hemicellulose and lignin.

In fermentation, the glucose content of hydrolysate is important, as *S. cerevisiae* catabolizes the hexoses in ethanol. This reaction occurs by means of the glycolytic pathway, whereby pyruvate (the product) is reduced to ethanol [25]. The resulting glucose concentrations after pretreatment with condition 5 and enzymatic hydrolysis with the Ctec2 enzyme are shown in Fig. 2. We highlight that the production of glucose from cellulose was higher in enzymatic hydrolysis ($P< 0.05$) than in acid pretreatment.

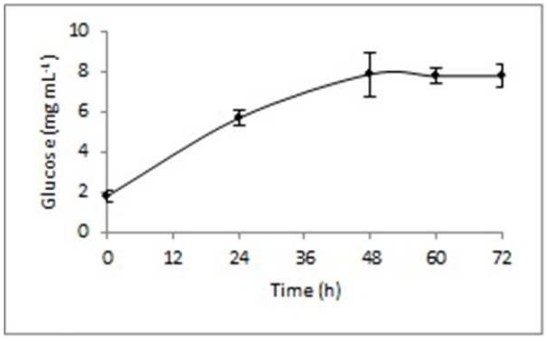

Fig. 2. Glucose concentration obtained from enzymatic hydrolysis with the CTec2 enzyme (40 FPU) from the biomass pretreated with 2% H_2SO_4/60 min/121℃

According to composition analysis in the sample, 37, 11.6 and 24.2% of cellulose, hemicellulose and lignin, were found respectively. After acid pretreatment, the remaining solid was close to 50%. Enzymatic hydrolysis was carried out with the remaining solid, resulting in 38.1% glucose (0.38 g g^{-1}). Determination of disaccharide presence was not possible using the selected chromatographic conditions, but these compounds may remain in the hydrolysate. Thus, the sugars available in the hydrolysate for the next step (*S. cerevisiae* fermentation) may be more than 38.1%.

Others researchers presented results near to 80% or more only from enzymatic hydrolysis [26, 27]. These results were very good because it was reached a total hydrolysis > 97% considering the acid pretreatment.

Another aspect that may have influenced the conversion was sample granulometry, as a granulometric size between 1.00 and 0.177 mm was used. The acid or enzyme attack was not efficient due to the large size of the particles. Particles ≥ 1.00 mm are easier to obtain on the farm because the farmers may have equipment for grinding. This grinding equipment does not break up the stalks into small enough particles for the high conversion of biomass. For this reason, future studies to scale up bioethanol production should consider evaluating cheaper equipment for sample comminution.

The best condition for the conversion of cellulose into glucose depends on temperature, granulometric size, reaction time and other variables. Further grinding of the sample and higher pretreatment temperatures are suitable options for achieving greater conversion. On the other hand, larger times (h) of enzymatic hydrolysis have not been proven to be the best option for increasing conversion.

The saccharification experiments allowed us to verify that sugar production and its subsequent conversion into ethanol is an option to add value to tobacco stalks.

3.3 Fermentation

The fermentation process was performed with hydrolysate prepared from the biomass of tobacco stalks that were pretreated with 2% sulfuric acid for 60 min and underwent enzymatic hydrolysis with CTec2 for 74 h.

Fig. 3 shows the ethanol concentrations during the 28 h of fermentation. After 10 h of fermentation, the ethanol production rate decreased.

The value obtained for ethanol from the fermentation of tobacco stalk biomass is in accordance with what was expected for glucose conversion to ethanol [28]. With *S. cerevisiae*, glucose conversion in ethanol was high, which makes high rates of saccharification important in this process.

According to Rios-Gonzalez, et al. [6], the maximum theoretical yield of ethanol from glucose (g g^{-1}) is 0.51. Based on the maximum ethanol content obtained from glucose after enzymatic hydrolysis, 120% (m/m) ethanol was obtained in these experiments. We assume that glucose and disaccharides were used by *S. cerevisiae* to grow, as well as others hexoses in the hydrolysate (mannose or galactose), because of this, more sugars were eligible to be converted to ethanol than the glucose analyzed. This result was close to 0.06 to 0.19 g of ethanol per g of tobacco stalks. The maximum ethanol production rate was approximately 3.22 g of ethanol per L per h from the hydrolysate. The result in g g^{-1} of biomass is less than what Martín, et al. [7] found with tobacco stalks subjected to steam explosion pretreatment at 205℃ for 5 or 10 min. In this same study, saccharification and fermentation were used simultaneously and maximum ethanol was 0.38-0.39 g of ethanol per g of tobacco stalks.

Another study, developed by Veramendi et al. [9, 10], obtained more sugars in the pretreatment plus enzymatic hydrolysis, therefore it was possible to obtain higher ethanol quantities in the fermentation compared to our results because they used transgenic plants. Thereby, it is confirmed that the saccharification conditions are the limiting step in ethanol production.

On the other hand, the results were better than those reported by Wang, et al. [11] that obtained ethanol from lignin from tobacco waste.

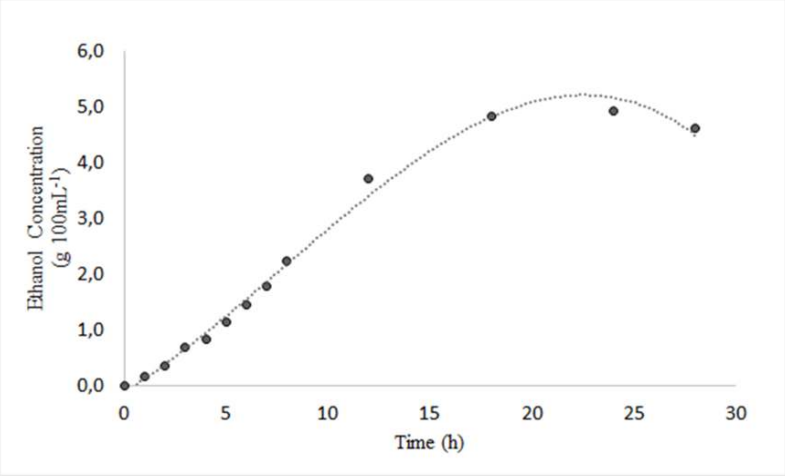

Fig. 3. Concentration curve of ethanol in fermentation of the hydrolysate of tobacco stalk biomass

4. CONCLUSIONS

It was concluded that the production of ethanol from tobacco stalks remaining in the field crop is possible. The exploitation of this agricultural waste for ethanol production may be another income source for small farmers that produce tobacco for cigarette purposes. The yield obtained for ethanol was 0.06 to 0.19 g of ethanol per g of milled and dried tobacco stalks. Further improvements of these results can be anticipated after an evaluation of the production conditions in a pilot study. Thereafter, modifications in acid pretreatment, enzymatic hydrolysis and fermentation may be necessary to improve the production conditions. This study, followed by an economic evaluation, may promote new ethanol production businesses from lignocellulose material in a region where residues from the tobacco industry (the stalks) are not well exploited and are generated in substantial quantities. Thereby, ethanol from tobacco stalks may be of great importance to agriculture and industry, as there is over 300,000 ha in Southern Brazil for tobacco production in which tobacco stalks remain in the soil, unused, after the leaves are harvested. The stalks are easy to harvest and separate and are available in great volumes in the region.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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