Autohydrolysis Pretreatment of Castor Plant Pruning Residues to Enhance Enzymatic Digestibility and Bioethanol Production

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Castor plant is used commonly for oil extraction and biodiesel synthesis. However, the residues during pruning are not being used effectively. These residues have the potential to be used as feedstock to produce bioethanol and other by-products. The present work assessed the eco-friendly autohydrolysis pretreatment of castor plant pruning residues at different severity factors (R_0), applying a range of temperatures from 100 °C to 200 °C. The hydrolysis of pretreated solids was carried out using a commercial cellulases complex at different solid and enzyme loadings. The enzymatic hydrolysate with a higher glucose concentration was further subjected to fermentation using *Saccharomyces cerevisiae* ATCC 4126. The results showed an efficient xylan hydrolysis (77.5%) and a preservation of glucan up to 83% in the solids pretreated at an R_0 of 5.78. The enzymatic hydrolysis of the pretreated solids at an R_0 of 5.78 showed a glucose release of 2.9-fold higher than non-pretreated material. In the hydrolysate fermentation, a maximum ethanol production of 50.5 g/L was achieved (equivalent to 6.4% v/v), corresponding to a conversion efficiency of 98% and a biomass-to-ethanol conversion yield of 93.0 g of ethanol per kilogram of feedstock.

Keywords: Autohydrolysis pretreatment; Bioethanol; Castor plant pruning residues; Enzymatic hydrolysis

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INTRODUCTION

Ricinus communis L. (castor plant) is a nonedible oil crop that is a member of the family Euphorbiaceae (Bauddh *et al.* 2015; Tropicos 2018). Castor plants tolerate a wide variety of weather conditions (they are commonly found as weeds alongside streams and polluted water) and have high biomass productivity (Vinayaka *et al.* 2017). Castor oil is conventionally used for a variety of industrial, cosmetic, and medical applications (Soni and Dhiman 2017); additionally, it is used for biodiesel production (Torrentes-Espinoza *et al.* 2017). During the period from 2004 to 2009, six industrial biodiesel plants were built in Mexico. Currently, due to the lack of a market and an insufficient supply of raw materials, these plants are not in operation (Montero *et al.* 2015). Recently, Mexico has started taking action towards the production and use of renewable and sustainable biofuels. In 2017, the first biodiesel plant from castor oil started operations in Mexico (FIRCO

2017). Castor plants grow quickly under conditions of good soil fertility, rapidly reaching a height of 3 to 6 meters, and at this height, pruning is recommended, generating a massive quantity of lignocellulosic residue (Vinayaka *et al.* 2017). Currently, these residues are not fully used, although they could be used as adsorbents in wastewater treatment processes (Santhi *et al.* 2010), polymeric composites (Vinayaka *et al.* 2017), biomass for pyrolysis (Kaur *et al.* 2018), and feedstock for bioethanol production (Bateni *et al.* 2014; Althuri *et al.* 2017). Hydrolysable sugars in the stem and leaf have the potential to be transformed into diverse important products such as bioethanol. In almost all second-generation (2G) processes for bioethanol production from lignocellulosic residues, there is a pretreatment step of the raw material to increase its digestibility and to improve the hydrolysis of the cellulose to obtain fermentable sugars (Díaz-Blanco *et al.* 2018; Jiang *et al.* 2020; Kumar *et al.* 2020). The use of these residues for ethanol production was first reported by Bateni *et al*. (2014), and castor stem seed cake and leaves were pretreated separately using sodium hydroxide at high temperatures. Althuri *et al*. (2017) reported the use of a blend of castor plant residues, *Saccharum spontaneum* L. and *Saccharum officinarum* L. Poaceae that was enzymatically pretreated (Tropicos 2018). The autohydrolysis pretreatment method uses only water. This method provides a simple, low-cost, and environmentally friendly pretreatment technology to obtain a material with high cellulose content by means of solubilization of hemicellulose (Ríos-González *et al.* 2017). To date, there have been no other reports of pretreated castor plant biomass using a hydrothermal pretreatment method (autohydrolysis or steam explosion). The use of these methods for pretreatment generates high value by-products (*e.g*., xylooligosaccharides) during depolymerization of hemicellulose in the liquid phase. The present work assessed the autohydrolysis pretreatment of castor plant pruning residues for ethanol production with the potential of widening the biorefinery utilization of this crop.

EXPERIMENTAL

Feedstock

Pruned biomass (stems, leaves, and petioles) was collected from a castor plant that had a height of 2.1 m and an age of 5 months from an experimental plantation for oil production of the Soil Department of the Universidad Autonoma Agraria Antonio Narro (UAAAN) located in the municipality of Saltillo, Coahuila, Mexico (latitude 25° 23' North, longitude 101° 00' West, altitude 1742 m). Before drying the pruned biomass, stems were manually cut to a size of 5 to 10 cm in length to facilitate further milling. The biomass was dried for further use in a Koleff Tray dehydrator (model KL10, Queretaro, Mexico) at 45 °C until the moisture content was less than 10% of the total weight. The dried mixture (material) was milled and sieved in a Retsch SM100 cutting mill (Retsch SM100, Retsch, Haan, Germany) to a particle size of 2 mm and subsequently stored at room temperature in hermetic containers.

Material Characterization

The moisture content of the material was determined in a thermobalance (OHAUS, Parsippany, NJ). Extractives were removed and determined as described by the National Renewable Energy Laboratory (NREL) analytical method NREL/TP-510-42619 (Sluiter *et al.* 2005). Structural carbohydrates (glucan and xylan) and lignin quantification was determined as described by Ríos-González *et al*. (2017). Ash and protein contents were

determined using the NREL/TP-510-42622 protocol (Sluiter *et al.* 2008) and by the Kjeldahl method (Morales-Martinez *et al.* 2017), respectively.

Autohydrolysis Pretreatment

Autohydrolysis pretreatments were carried out in a 3.75 L agitated stainless steel reactor (model 4551, Parr Instruments, IL, USA) equipped with a temperature controller (model 4838, Parr Instruments). The reactor was loaded with 200 g (dry weight base) of material and 1.2 L of distilled water, resulting in a solids loading of 16.6% (w/v). The resulting mixture was hydrated for 30 min before pretreatment at room temperature. All experiments were conducted in triplicate and under agitation at 200 rpm.

The severity factors (R_0) of the autohydrolysis pretreatment were calculated considering the heating up and cooling down time, plus the isothermal regime established according to Eq. 1 proposed by Chornet and Overend (2017),

$$R_0 = [R_{0\,HEATING} + R_{0\,ISOTHERMAL\,PROCESSING} + R_{0\,COOLING}] = \\ = \left[\int_0^{t_{MAX}} exp\left(\frac{T(t)-T_{REF}}{\Omega}\right) dt + \int_{IsoI}^{IsoF} exp\left(\frac{T_{(t)}-T_{REF}}{\Omega}\right) dt + \int_0^{t_{MAX}} exp\left(\frac{T'(t)-T_{REF}}{\Omega}\right) dt\right] \quad (1)$$

where t_{MAX} is the time (min) needed to achieve the maximum temperature (T_{MAX}, °C); $T(t)$ and $T'(t)$ (°C) are the temperature profiles in heating and cooling, respectively; ω and T_{REF} are parameters that also have been reported (Ω = 14.75 °C; T_{REF}= 100 °C), and Iso_I and Iso_F is the time at which the isothermal stage on treatment is initiated and terminated, respectively (min).

The reactor was heated to the desired temperature described in Table 1 (140 °C, 160 °C, 180 °C, and 200 °C and maintained in isothermal regime for 15 min). A control was established at a severity factor of 1.14 at 100 °C for 10 min. Once the desired severity factor was accomplished, the reactor was immediately cooled. The reactor was opened until the temperature decreased to 80 °C (Rios-Gonzalez *et al*., 2017). The pretreated material was separated by filtration, washed with a water volume of 30 times the volume of the material to eliminate the presence of inhibitors adhered to the solids, and stored at 4 °C before performing the enzymatic hydrolysis tests. The contents of glucan, xylan, and lignin in the solid fractions were determined as described in the previous section. The liquid fraction was analyzed by high-performance liquid chromatography (HPLC) to determine the concentration of sugars (glucose and xylose).

Enzymatic Hydrolysis

Cellic® CTec3 (kindly provided by Novozymes A/S, Denmark) enzyme complex was used to hydrolyze the pretreated solids (FPase = 217 FP units/mL). Processing was done according to the NREL/TP-510-42629 protocol (Selig *et al.* 2008). The experiments were carried out in 125 mL Erlenmeyer flasks (working volume of 30 g) using solids and enzyme loadings of 10% (w/v) and 25 FPU (Filter Paper Units)/g of glucan, respectively, in 0.05 M sodium citrate buffer at a pH of 4.8. Reaction flasks were incubated at 50 °C in an orbital shaker (New Brunswick TM124/24R, NY, USA) at 200 rpm for 72 h. Samples were centrifuged, filtered, and analyzed by HPLC to quantify the glucose concentration. The enzymatic hydrolysis yields were expressed as the ratio between the amount of glucose released during hydrolysis and the initial amount of structural glucose present in the pretreated material (hydrolysis efficiency) or in the raw material (Table 1). The glucan (highest value obtained) and xylan (lowest values obtained) content in the solid fraction and the enzymatic hydrolysis yield (highest value obtained) were selected to be used in the

enzymatic hydrolysis stage.

Enzymatic Hydrolysis (Experimental Design)

A 3^2 full randomized factorial design was used in the present study for the pretreated solid selected. In this design, two factors were assessed at 3 levels each: solids loading (%, w/v) and enzyme loading (FPU/g of glucan), as shown in Table 2. The amount of glucose released was selected as the independent variable. Three controls were maintained using material with no pretreatment and with an enzyme loading of 25 FPU/g glucan at three levels of solid loadings.

Ethanol Production

The hydrolysate obtained under optimal conditions was centrifuged at 5,082 rcf for 15 min in a Thermo Scientific centrifuge (Haraeus Megafuge™ 16 R, Rockford IL, USA). Subsequently, the hydrolysate was supplemented with: yeast extract (10 g/L), monopotassium phosphate (1.17 g/L), calcium chloride (0.09 g/L), magnesium sulfate (0.36 g/L), and ammonium sulfate (4.14 g/L) and fermented using *Saccharomyces cerevisiae* ATCC 4126 in a 125 mL Erlenmeyer flask with a working volume of 50 mL. The medium was supplemented with 15 mL/L of a salts solution containing: sodium chloride (1.26 g/L), cupric sulfate (0.26 g/L), ferrous sulfate (0.22 g/L), manganese chloride (0.12 g/L), and zinc chloride (0.32 g/L). The pH of the hydrolysate was adjusted to 5.5 with 2 M NaOH before inoculation. The flasks were incubated in an orbital shaker at 35 °C and 150 rpm for 24 h. Samples were taken at 0, 2, 4, 6, 8, 10, 12, 18, and 24 h for ethanol and glucose quantification by HPLC. The enzymatic hydrolysis yield is expressed as the relationship between the amount of glucose released during processing and the initial amount of glucan present in the pretreated solids. The ethanol yield is reported as a percentage of the theoretical yield assuming all of the potential glucose present can be fermented, with a maximum theoretical ethanol yield of 0.51 g ethanol/g glucose.

Analytical Methods

The glucose, xylose, and ethanol were determined using an HPLC unit (Agilent 1260 Infinity, Santa Clara, CA, USA) equipped with a refractive index detector at 45 °C, using an Agilent Hi-Plex H column at 35 °C (7.7 × 300 mm) and 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min.

Data Analysis

All experiments were performed in triplicate, and the average values are reported. An analysis of variance (ANOVA) was conducted along with Fisher's F test with a p value of < 0.05 (Minitab® version 17, Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Material Composition

The composition analysis of the raw castor plant pruning residues (dry basis w/w) showed that polysaccharides content was 42.1%, where the main component is glucans at 32.3%, followed by the extractives (23.3%), lignin (23.3%), xylans (9.78), ash (5.73%), and proteins (3.68%). The glucan and xylan content were below those reported by Vinayaka *et al.* (2017) of 45% and 30%, respectively, with the difference being that only

the stems were used. The stems have a higher content of structural carbohydrates (glucan and xylan) compared to the leaves and petioles (data not shown, obtained in preliminary studies); in addition, the farming conditions of each crop play an important role in these findings. Van der Weijde *et al*. (2017) mentioned that longer exposure to drought stress challenges the plants to modify their cell walls to sustain growth under conditions with reduced water potential. The extractives content is similar to other values found in the literature (Mukhopadhyay *et al.* 2011), which are commonly found secondary metabolite compounds with properties beneficial to health (Alugah and Ibraheem, 2014; Soni *et al.* 2017). Some of these secondary metabolites are soluble in water and can be extracted by hydrothermal treatments such as autohydrolysis (at low temperatures < 100 °C) as a pre-treatment stage with high temperatures.

Autohydrolysis Pretreatment

The composition of castor plant pruning residues after autohydrolysis pretreatment at the different severity factors assayed are shown in Table 1. An increase in the temperature from 100 °C to 160 °C only produced an increase in 2.2% of xylan hydrolysis. At 180 °C and 200 °C, the xylan hydrolysis was higher (44% and 77.5%, respectively). The glucan content in the recovered solids after all pretreatments was higher compared to the untreated material (increasing from 34.2% to 50.3%). In experiments carried out at the maximum SF assessed (3.37), 83% of the glucan content remained in the solid fraction. Additionally, in all experiments, lignin was not significantly solubilized during the autohydrolysis pretreatment. The results obtained in the present study are similar to previous reports (Morales-Martínez *et al.* 2017; Ortiz-Méndez *et al.* 2017; Ríos-González *et al.* 2017), in which xylan was hydrolyzed during pretreatment, while the glucan and insoluble lignin were retained in the solid fraction.

As expected, the recovered solids (solids remaining after pretreatment, based on the initial biomass weight) decreased when the SF increased. Thus, the recovered solids ranged from 53.4% to 94.5%, corresponding to the highest (SF = 3.37) and the lowest severity factor (SF = 1), respectively. This loss is attributed to the extractives and the xylan that were removed during autohydrolysis pretreatment.

The enzymatic hydrolysis of the pretreated solids also showed an increase as the SF increased, obtaining higher hydrolysis yields that ranged from 52.0% to 97.8% with SFs of 1.0 to 3.37, respectively. This behavior was also observed by Bateni *et al*. (2014), who reported an improvement in the hydrolysis of castor plant residues in samples pretreated with 8% (w/v) NaOH solution at a high temperature for a longer time (100 °C for 60 min) that caused a higher degree of hydrolysis of some parts of the hemicellulose, which mainly consist of xylan. Because enzymatic pretreatment presents a higher specificity and does not generate high concentrations of inhibitory products, this process is regarded as slow compared to physical and chemical pretreatment methods. This was mentioned by Mukhopadhyay *et al*. (2011), who reported a maximum delignification of castor plant residues after 6 h of enzymatic pretreatment. Finally, after analyzing the results obtained (considering glucan preservation, xylan removal and hydrolysis yield), the solids pretreated at an SF of 3.37 (200 °C for 15 min) were selected for the following process of enzymatic hydrolysis.

Table 1. Composition of Solids and Liquid Fractions after of Autohydrolysis Pretreatment of Castor Plant Pruning Residues

Temperature (°C)	100	140	160	180	200
R₀	1.14	3.25	4.03	4.84	5.78
Recovered solids (%)	94.53 ± 1.9	89.15 ± 1.3	87.30 ± 1.7	63.45 ± 0.91	53.35 ± 1.1
Loss solids (%)	5.47 ± 1.9	10.85 ± 1.3	12.70 ± 1.7	36.55 ± 0.91	46.65 ± 1.1
Solids composition (g / 100 g_{DWM})[a]					
Glcn	34.23 ± 1.2	36.32 ± 1.4	37.08 ± 1.3	46.92 ± 0.96	50.28 ± 1.6
Xyln	10.24 ± 0.38	10.85 ± 0.24	10.84 ± 0.19	8.57 ± 0.32	4.08 ± 0.15
KL	24.61 ± 0.31	26.1 ± 0.52	26.65 ± 0.26	36.65 ± 0.43	44.94 ± 0.71
Solid fraction removed (g / 100 g_{DWM})[a]					
Glcn	0 ± 0	0 ± 0	0 ± 0	8.1 ± 0.93	17 ± 1.35
Xyln	0 ± 0	0 ± 0	2.2 ± 0.13	44 ± 0.61	77.5 ± 0.62
KL	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Liquid composition (g/L)					
Glc	0 ± 0	0 ± 0	0 ± 0	0.56 ± 0.3	0.94 ± 0.45
Xyl	0 ± 0	0 ± 0	0.04 ± 0.01	0.62 ± 0.09	1.60 ± 0.19
FA	0 ± 0	0.55 ± 0.02	0.8 ± 0.04	1.85 ± 0.05	1.75 ± 0.09
AA	0 ± 0	0.11 ± 0.07	0.36 ± 0.04	2.2 ± 0.01	4.5 ± 0.08
FUR	0 ± 0	0 ± 0	0 ± 0	0.11 ± 0.01	0.49 ± 0.02
Enzymatic hydrolysis (72 h)					
Glucose (g/L)	23.43 ± 0.34	27.78 ± 0.16	30.35 ± 0.81	43.51 ± 0.25	54.06 ± 0.72
Hydrolysis Yield (%)	51.99 ± 0.34	61.44 ± 0.16	74.4 ± 0.81	84.31 ± 0.25	97.77 ± 0.72

[a]: DWM: dry-weight mass. Solid composition represent the amount of solids recovered after the pretreatment at different severity factors (SF): KL: Klason lignin; Glcn: glucans; Xyln: xylans. Liquid composition represents the amount of components released: Glc: glucose; Xyl: xylose; FA: formic acid; AA: acetic acid; and FUR: furfural; HMF was not detected

Enzymatic Hydrolysis

Table 2 shows the results obtained for the enzymatic hydrolysis of the pretreated material using Cellic® CTec3 at different solids and enzyme loadings. The increase in the solids loading from 10% to 20% significantly increased the release of glucose (52.2 g/L to 99.9 g/L respectively). Figure 1 shows that the increase of solids loading caused a slight decrease in the hydrolysis yield (94.5% to 90.3%, respectively).

Table 2. Enzymatic Hydrolysis of the Pretreated Material at Different Solids and Enzyme Loadings

Experiment No.	Solids Loading (% w/w)	Enzyme Loading (FPU/g glucan)	Glucose (g/L)	Hydrolysis Yield (%)
1	20	25	103.76 ± 0.49	93.81
2	20	20	100.46 ± 0.57	90.83
3	20	15	95.52 ± 1.34	86.36
4	15	25	79.19 ± 0.82	95.46
5	15	20	76.91 ± 0.99	92.71
6	15	15	73.69 ± 0.34	88.83
7	10	25	54.36 ± 0.93	98.31
8	10	20	52.24 ± 0.49	94.47
9	10	15	50.19 ± 0.77	90.76
US (Control 1)	20	25	35.11 ± 0.82	31.75
US (Control 2)	15	25	28.65 ± 0.72	34.54
US (Control 3)	10	25	19.96 ± 0.24	36.10

US: Untreated Solid

The maximum glucose released (103.8 g/L) was observed in the experiments with the highest values of solids and enzyme loadings (20% and 25 FPU/g glucan, respectively). Under these conditions as previously mentioned but using the nonpretreated material (Control 1), the glucose released (35.1 g/L) and the hydrolysis yield (31.8%) were 2.9-fold lower. Similar results were reported by Mukhopadhyay *et al*. (2011), with a 2.68-fold increase in reducing sugars release yields during enzymatic hydrolysis of castor plant biomass pretreated by laccases compared with non-pretreated castor plant biomass. Bateni *et al*. (2014) carried out the hydrolysis of castor plant residues (stem, leaves and seed cake) and reported the results separately, mentioning that an improvement was observed in the hydrolysis yield of pretreated material with alkali at 100 °C for 60 min (82.9%, 35.4 and 61.3% for stem, leaves and seed cake, respectively) compared to non-pretreated material (36.1%, 18.2% and 7.6% for stem, leaves and seed cake, respectively).

According to Fig. 1 and the ANOVA analysis (Table 3), the factor with more significance was solids loading, where at high levels, the higher hydrolysis yield was obtained. For enzyme loading, its significance was low in all of the levels evaluated. This could represent an advantageous feature of our process.

Table 3. Analysis of Variance

Factors	DOF	Sums of squares	Variance	F-Ratio	p
Solids loading	2	6812.128	3406.064	3039.395	0.000000
Enzyme loading	2	107.753	53.877	48.077	0.000016
Solids vs Enzyme loading	4	9.128	2.282	2.036	0.172600
Other/Error	9	10.086	1.121		
Total	17	6939.095			

$p < 0.05$ = Higher factor significance

These results show that by using low levels of enzyme, high hydrolysis is obtained. In the same regard, high solid loading favor hydrolysis. This represents low cost in enzymes and high amount of substrate. Also, the results show that at even higher solids loading, even higher hydrolysis yield may be obtained.

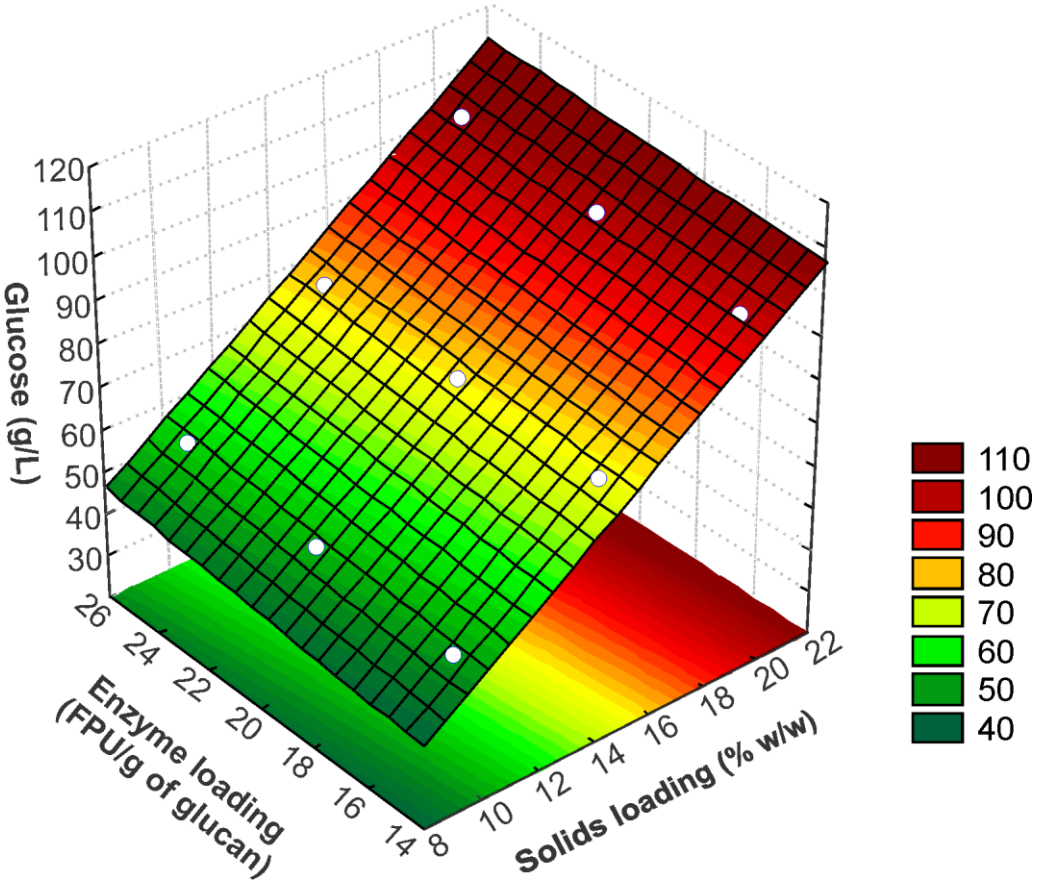

Fig. 3. Effect of the variables in enzymatic hydrolysis of castor plant

In the actual results of the evaluated treatments (Table 2), even though the highest glucose release observed was in the cases where the highest enzyme loading was used (Experiments 1, 4, and 7), the difference in glucose release on average was only 5.97 g/L compared to the experiments with the lowest enzyme loading used (Experiments 3, 6, and 9). This slight difference can be attributed to the blockage of active sites of cellulase due to the high content of lignin present in the pretreated material (Althuri *et al.* 2017) or by the inhibition of the enzymatic complex due to hydrolysis products, such as glucose and cellobiose. According to Morales-Martinez *et al*. (2017), an increase of 50% in enzyme loading could be justified only if the increase in the hydrolysis yield was greater than 6%. Therefore, the enzyme loading can be optimized to provide the maximum glucose concentration at the lowest unit cost (Wang *et al*. 2012). With the goal of obtaining the highest possible ethanol yield (0.51 g of ethanol per g of glucose), the hydrolysates with the highest glucose concentration (Experiment 1) were used in the subsequent fermentation stages.

Fermentation of Hydrolysates

Figure 1 shows the ethanol production and glucose consumption from hydrolysates of castor plant pruning pretreated by autohydrolysis. The maximum ethanol produced was 50.5 g/L (equivalent to 6.4% v/v) at 12 h of incubation, and at this same time, glucose was totally consumed. The ethanol concentration obtained corresponds to an ethanol yield of ($Y_{E/G}$) 0.50 $g_{ethanol}/g_{glucose\ consumed}$ and a conversion efficiency (CE) of 98% according to the theoretical value. The biomass to ethanol conversion yield ($Y_{E/B}$) was 93.0 g of ethanol per

kilogram of raw castor plant pruning residues. The results obtained in the present work regarding the $Y_{E/B}$ were greater than those reported by Bateni *et al*. (2014), with 63 g of ethanol per kilogram of castor stems pretreated with NaOH at 100 °C for 60 min. In another report (Althuri *et al.* 2017), a higher ethanol concentration was produced (62.0 g/L equivalent to 7.86 v/v) compared to the results of the present work; however, in this case, the material used by the authors had a composition of 40% of *Saccharum spontaneum* L. and *Saccharum officinarum* L. These two species have been reported to have a high content of glucan, which can explain the greater sugar and ethanol production in the hydrolysis and fermentation stages, respectively. Further research will be focused on maintaining the high hydrolysis yield obtained in the present work and improving the processes for extraction and conservation of biomolecules of interest.

CONCLUSIONS

1. The autohydrolysis pretreatment of castor plant pruning residues was found to be an efficient method. This pretreatment favored enzymatic hydrolysis, obtaining high yields.
2. Castor crops have potential to be used as raw material for a biorefinery process using the whole plant. The main objective of the autohydrolysis process is to avoid the use of chemicals, allowing for a higher preservation of the bioactive molecules of interest present in the extracts.
3. The pretreatment should be carried out at temperatures under 180 °C and with a reaction time equivalent to an SF of 3.37.
4. The ethanol production was efficient, accumulating up to 50.5 g/L in 12 hours with the glucose available was consumed in its totality adjusting to the theoretical yields for ethanol production. This result confirms the viability of the biomass as an extended use of the material regarding biofuels production.

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