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Ethanol From Cashew Apple Bagasse By Enzymatic Hydrolysis

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The advantages of using ethanol as fuel in its hydrated form as a substitute for gasoline or mixed with gasoline as anhydrous ethanol are numerous, with reduction of harmful emissions and reduced emissions of greenhouse gases. This work aims to transform the energy potential available in the cashew apple bagasse by enzymatic processes for obtaining hydrated ethyl alcohol and to study the enzymatic hydrolysis of lignocellulosic cashew bagasse raw-material (*Anacardium Occidentale* L.) and bioethanol production from the hydrolyzed liquor. The raw materials used in this study were cashew bagasse and sugarcane bagasse (residue used only for comparison with cashew bagasse). Evaluating the process of enzymatic conversion of cellulose into fermentable sugars, the enzymatic hydrolysis performance with 7 FPU and enzyme load of 3.5 FPU/g showed better efficiency for cashew bagasse in a time of 12 h; however, sugarcane bagasse was more efficient in time of 48 hours. Regarding the process of alcoholic fermentation of the cashew bagasse hydrolyzed liquor, it is evident that all the sugar was consumed and almost all ethanol was produced in an 8 h process, so the best test for the efficiency of the fermentation process (90 % conversion) is to operate with concentrations of 10 g/L, 0.6 g/L and 0.12 g/L of yeast, source of N and P, respectively.

1. Introduction

Enzymatic hydrolysis presents a number of advantages over chemical hydrolysis. The main advantages are specificity, control of the hydrolysis degree, mild action conditions (mild temperature) and minimum formation of byproducts, and main disadvantages are the high costs of the enzymatic complex and the need for pretreatment to achieve efficient conversion rates (Clemente, 2000).
Among the fruits grown in Northeastern Brazil, cashew deserves attention due to its socioeconomic importance in the country. It is estimated that about 80 % of the pulp, i.e., the cashew stalk is wasted. Therefore, 1.9 Mt of this food rich in nutritional content are wasted (Alcântara et al., 2010).
Brazil has the conditions to be the main recipient of investments in the production and use of bioenergy segment, with the environment as its greatest asset, emerging as a major producer of renewable raw materials such as agricultural crops, especially sugarcane and being a pioneer in the production of ethanol biofuel. The biotransformation of such waste by enzymatic processes for ethanol production necessarily requires careful scientific and technology study in the pursuit of kinetic parameters to adjust and control the reactions involved. Thus, various substrates have been used as a means for obtaining ethanol such as sugarcane bagasse (Rueda et al., 2010), sisal (Lima et al., 2013) and others (Chin et al., 2011; Yi et al., 2009 and Vancov et al., 2005).
Researchers (Rueda et al., 2010) studied the pretreatment, and also, the enzymatic hydrolysis of sugar cane bagasse with phosphoric acid and diluted sulfuric acid to produce fermentable. Evaluated through experimental design the influence of variables: concentration of sulfuric acid and phosphoric acid (0.5 to 3.5 %) and reaction time (15-180 min) in the pretreatment with dilute acids and enzymatic hydrolysis by checking the best values obtained for glucose. The pretreatment temperature and solids concentration remaining constant at 130 °C and 10 % pulp. The maximum concentration achieved for glucose was 404.5 mg

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glucose/mg bagasse pretreated with phosphoric acid and 414.9 mg of glucose /g bagasse pretreated with sulfuric acid. Studying the production of bioethanol from bagasse hydrolyzate using sisal (Lima et al., 2013) found of 92 % efficiency for ethanol fermentation, obtained by industrial yeast *Saccharomyces cerevisiae*. The authors were able to improve the efficiency of the pretreatment using a temperature of 105 °C with 3% H_2SO_4, mainly pentoses releasing the prehydrolyzate liquor. Acid hydrolysis of bagasse was more efficient when operated using 3 % H_2SO_4 at a temperature of 160 °C at a pulp: acid ratio of 1:10, yielding the maximum release of glucose liquor 5.8 g / L.

This paper aims to transform the energy potential available in the cashew apple bagasse by enzymatic processes for obtaining hydrated ethyl alcohol and to study the enzymatic hydrolysis of lignocellulosic cashew bagasse raw-material (*Anacardium occidentale* L.) and sugarcane bagasse for comparison purposes and bioethanol production from the hydrolyzed liquor.

2. Material and methods

2.1 Raw material

The raw materials used in this research were cashew bagasse and sugarcane bagasse (used for comparison with cashew bagasse).

Cashew bagasse was purchased from FRUTNAT juice production industry, located in the city of Campina Grande, Paraíba, which was transported to the processing site, where it was submitted to two initial washes with hot distilled water at temperature around 50ºC and time of 20 min for each wash. After washing with hot water, cashew bagasse was submitted to two washings with distilled water at room temperature for the leaching of remaining sugars during juice extraction up to achieving ºBrix zero.

Sugarcane bagasse was processed in mill for extraction of sugarcane juice. It was also washed in distilled water to achieve ºBrix zero. This bagasse was used in the enzymatic hydrolysis for comparative purposes with cashew bagasse. Then, the material was removed, ground and sieved to 48 mesh to reduce the particle size, being subsequently vacuum packed in polyethylene bags and stored in sealed boxes for later use.

2.2 Chemical Composition of cashew bagasse and sugarcane bagasse Seeking to compare the physicochemical characteristics of cashew bagasse and the most abundant lignocellulosic raw material in Brazilian agribusiness (sugarcane bagasse), analyses of pH, moisture and total soluble solids (Brazil, 2005), cellulose and hemicellulose (Xu et al., 2006), lignin (Rose and Vieira, 2007), extractives (Lima et al., 2013) of these raw materials were performed.

2.3 Enzymatic hydrolysis

With material produced under the best acid prehydrolysis conditions (Rocha et al., 2011) study on the enzymatic hydrolysis of cashew bagasse pretreated with acid was carried out. Enzymatic hydrolysis with sugarcane bagasse under the conditions reported by Rueda et al. (2010) was also held for comparison purposes. The hydrolysis of cashew bagasse was an adaptation of methodologies used in the enzymatic hydrolysis of sugarcane bagasse.

2.3.1 Acid pretreatment and enzymatic hydrolysis of biomasses

The acid pretreatment used 10 g of cashew apple bagasse on a dry basis in each sample, which was treated with solutions of acids (phosphoric acid 85 % purity and sulfuric acid 95 % purity VETEC tag / PA with concentrations of 0.5, 2.0 and 3.5 %). Then, 100 mL of each solution with the sample were placed in a stainless steel MAINTEC FORNOS INTI reactor with thermal controller FE50RP. Reactions were conducted at temperature of 130 °C and times of 15, 97, 180 min.

After acid treatment, the bagasse was washed with distilled water up to reaching neutral pH. Then, it was filtered and the retained solid was treated with 1.5 % NaOH at 100 °C. Again, the solid was washed with distilled water up to reaching neutral pH. After acid and basic pre-treatment, enzymatic hydrolyses were carried out varying the enzymatic activities. The cellulase used was produced in a microbial way using *Aspergillus niger* C1184-5KU (Sigma-Aldrich).

For enzymatic hydrolysis, two grams of pretreated cashew bagasse were hydrolyzed with commercially available enzyme. The pretreated cashew bagasse was placed in a 250 mL Erlenmeyer together with 45 mL of buffer solution (2 g citric acid /7 g sodium citrate), pH 4.8, in which enzyme was dissolved to have activities in 7 FPU, or enzyme load of 3.5 FPU/g. Enzymatic hydrolysis for saccharification occurred in bagasses contained in Erlenmeyer flasks stored at T = 50 °C in an shaker incubator (Marconi MA-420), stirring at 150 rpm. The hydrolysis times were 48 and 72 h to determine which showed better response, after acid pretreatments in terms of glucose.

2.3.2 Sugar and ethanol contents

Sugar and ethanol contents were determined by High Performance Liquid Chromotography (HPLC) with a ProStar 210 (Varian) pump, manual injector of 20 µL l loop; ProStar 356 (Varian) refractive index detector and 284 nm UV/visible (Aldehydes), Hi-Plex H (300 mm x 7.7 mm, Varian) stainless steel analytical column. Operating conditions were as follows: Column temperature 40 °C. Mobile phase: MilliQ water with flow rate of

0.6 mL/min; Analysis time: 15 min and 60 min for sugar and ethanol contents, respectively. Internal standard sugar solutions: glucose and ethanol (Sigma 99.99 % HPLC grade), were used to quantify liquor components.

2.4 Alcoholic fermentation of the cashew bagasse saccharified liquor

2.4.1 Inoculum preparation

About 10 g of selected active FT858 yeast were used (GIASA- distillery producing ethanol industry Paraíba / Brazil), *Saccharomyces cerevisiae* strain. In 1 L of distilled water, 100 g of sucrose sugar and the amounts of nutrients 0.6 and 0.12 ammonium sulfate and potassium phosphate, respectively (medium sterilized by autoclaving). The process of yeast reactivation has begun. With the aid of a pump, the system was activated and fed with substrate every half hour during two hours under constant aeration with the objective of providing oxygen, thereby avoiding increased ethanol production and enabling cell reproduction.

After the cell mass increase, the inoculum was centrifuged in excelsa II centrifuge model 206 BL at 1500 rpm and placed in 100 mL buckets totaling 400 ml every 5 min of centrifugation with the aim of separating suspended solids in the medium.

2.4.2 Inoculation of the microorganism in the hydrolyzed liquor

After the inoculum preparation steps, the microorganism (industrial yeast *Saccharomyces cerevisiae,* gently provided by GIASA), and the enzymatically hydrolyzed liquor was supplemented with NH_4SO_4 (ammonium sulfate) and KH_2PO_4 (potassium phosphate). Was inoculated in the hydrolyzed liquor, thus beginning the ethanol production step in 250 mL Erlenmeyer flasks using 30 g of selected and activated *Saccharomyces cerevisiae* already centrifuged and filtered in which 60 mL of liquor were used. Alcoholic fermentation took place in Shaker Marconi incubator model MA-420 at temperature of 30ºC and 150 rpm.

2.4.3 Alcoholic fermentation kinetic profile

Alcoholic fermentation kinetic parameters were calculated (% conversion, yield and theoretical yield of substrate conversion into ethanol).

The % conversion of fermentations represented by the conversion factor of total sugars into ethanol, the fermentation productivity, expressed as mass of product formed per unit of time per unit of volume (g/L h) and the theoretical yield values ($Y_{P/S}$), were calculated according to Eqs (1), (2) and (3), respectively.

$$\%\text{Conversion} = \frac{P_i}{Si \text{ x } 0.511} \text{x} 100 \quad (1)$$

$$Q_p = \frac{P_i - P_0}{t_f} \quad (2)$$

$$Y_{P/S} = \frac{(P_f - P_0)}{(Si - S_f)} \quad (3)$$

Where: P_f - ethanol concentration in the final fermentation time (g/L); P_0 - ethanol concentration in the beginning of the fermentation process (g/L); S_i - glucose concentration the beginning of the fermentation process (g/L); S_f - glucose concentration in the final fermentation time (g/L); t_f - final fermentation time, corresponding to the maximum ethanol concentration in the fermented broth (h).

2.5 Statistical analysis for the comparison of the means of enzymatic hydrolysis

The Tukey test with 1 % probability was applied in the mean glucose levels in the hydrolyzed liquor, in the times studied.

2.6 Factorial experimental design of the study on the alcoholic fermentation of enzymatically hydrolyzed liquor

The 2^3 factorial design with 3 central points was used to investigate the influence of the input variables (initial yeast concentration, initial concentration of source of nitrogen and initial concentration of source of phosphorus) on the alcoholic fermentation kinetic parameters (% conversion, volumetric yield and theoretical yield of the process).

3. Results and discussion

3.1 Physicochemical characterization of cashew and sugarcane bagasse

Table 1 shows the physicochemical compositions of cashew and sugarcane bagasse.

The percentage of cellulose and lignin in the bagasse used in the present study was 38.00 and 22.00 %, respectively. Comparative data show similar values, indicating that the percentage of cellulose is lower compared to that obtained in literature, 42.8 %, 22.1 %, which used sugarcane bagasse (Gouveia et al., 2009). Alcântara et al. (2010) studied the production of pectinase using cashew bagasse and obtained in the physicochemical characterization of dry bagasse pH of 3.66, a value lower than that obtained in the present

study (4.76). In the case of sugarcane bagasse, the pH found by (Gouveia et al., 2009) of 6.88 % was much higher than value found in this study (5.46).

Table 1: Physicochemical characterizations of cashew and sugarcane bagasse

Analyses	Cashew bagasse	Sugarcane bagasse
pH	4.76 ± 0.05	5.46 ± 0.05
Moisture%	7.5 ± 0.20	3.08 ± 0.02
Cellulose %	34.93 ± 0.55	38.00 ± 0.02
Lignin %	23.53 ± 0.37	22.00 ± 0.05
Extractives %	15.13 ± 0. 005	4.48 ± 0.05
Soluble Solids %	0.0	0.1
Hemicellulose%	8.20 ± 0.07	6.0 ± 0.10

As can be seen in Table 1, comparing cashew bagasse and sugarcane bagasse, 7.5 % and 3.08 % respectively, for the moisture content, it was found that there is higher moisture content in the cashew bagasse than in sugarcane bagasse, about 2.4 times.
The work (Gouveia et al., 2009) found in sugarcane bagasse without pretreatment, value higher than that of this work, 42.8%. The content of extractive compounds in cashew bagasse found in this study was 15.13%. This value is above results found in sugarcane bagasse, which was 4.48 %. This is due to the characteristic of the material, since cashew bagasse presents more extractive compounds (waxes, tannins, among others) than sugarcane bagasse.
As can be seen in Table 1, the soluble solids content of cashew and sugarcane bagasse showed similar values, between 0.0 and 0.1 °Brix respectively. Washes made in cashew apple and sugarcane waste removed the soluble solids. Once characterized (Table 1), it could be inferred that cashew bagasse is an interesting plant biomass for cellulosic ethanol production, being similar to sugarcane bagasse.

3.2 Enzymatic hydrolysis

This work was aimed at studying the enzymatic hydrolysis of the lignocellulosic material, cashew bagasse pretreated with 0.5 % sulfuric acid, 10 % (w/v), bagasse, 130 °C, and reaction time 15 min (Rose and Vieira, 2007). The verification of the enzymatic hydrolysis was carried out by observing the release of glucose with time in the reaction medium (hydrolyzed liquor).
Table 2 shows the monitoring of glucose concentrations of enzymatic hydrolysis processes under conditions of 150 rpm agitation, 50 °C in citric acid - sodium citrate buffer solution pH = 4.8 ± 02.
Table 2 shows that for an enzymatic activity of 5.25 FPU of cellulase, the amount of glucose produced was 25,103.95 mg/L of cashew bagasse and 22,201.02 mg/L of sugarcane bagasse in a reaction time of 72 h. The maximum glucose release in this enzymatic hydrolysis was observed for substrate cashew bagasse. With increasing addition of cellulase for activity of 7 FPU, the best glucose concentration values in the reaction medium for cashew bagasse was 24,573.36 mg/L at 12 h of hydrolysis and for sugarcane bagasse it was 24,011.00 mg/L in 48 h of hydrolysis. Rueda et al. (2010) studied the glucose production by enzymatic hydrolysis of sugarcane bagasse pretreated with two dilute acids and obtained the best result with sulfuric acid, with maximum release of 414.9 mg glucose/g bagasse.

Table 2: Glucose concentration of hydrolysis processes

Time (hours)	Cashew bagasse glucose (mg/L)	Sugarcane bagasse glucose (mg/L)
	5.25 FPU cellulase*	
12	18540.59d	19653.79b
24	20200.82c	17565.29d
48	22283.81b	18104.84c
72	25103.95a	22201.02a
	7 FPU cellulase*	
12	24573.36a	20922.51d
24	24444.17b	22466.68c
48	22973.63c	24011.00a
72	21274.73d	22841.33b

*Means followed by same letter in the column do not differ according to the Tukey test at 1 %.

Assessing the enzymatic conversion of cellulase into fermentable sugars, the enzymatic hydrolysis performance with 7 FPU showed for cashew bagasse, in a time of 12 h, glucose concentration in the

hydrolyzed liquor compared with the maximum possible (41.9 g/g cellulose) of 60.0%, while for sugarcane bagasse, 55.0 % of the maximum (43.6 g/g cellulose) in a time of 48 h. To increase the hydrolysis efficiency, it will be necessary to increase the enzyme addition, enzymatic activity, which should be above 7 FPU, keeping the amount of bagasse in 2 g, since in this study, the maximal enzyme load used was 3.5 FPU/g of pretreated bagasse.
The lignocellulosic material from the cashew apple bagasse shows promising potential for obtaining cellulosic ethanol. Sugarcane bagasse is a material already well studied in Brazil and has proven its potential in obtaining bioethanol (Rueda et al., 2009; Wei-Hsin et al., 2012).

3.3 Study of the alcoholic fermentation kinetics

Enzymatically hydrolyzed liquor was used in the study of the alcoholic fermentation kinetics.
Table 3 shows the design experiment matrix with the results of the kinetic parameters. According to the described methodology, the fermentation kinetics was performed with the enzymatically hydrolyzed liquor, using different concentrations of yeast, NH_4SO_4 and KH_2PO_4 (as sources of N, P) for the respective experiments. With the results of ethanol production and substrate consumption in the fermentation medium, the kinetic parameters of theoretical yield, ethanol % conversion and productivity at the peak of highest ethanol concentration were obtained.
Analyzing the results shown in Table 3, found that the experiment 9 presents the best values for the kinetic parameters. That all the sugar is consumed in 8 h and virtually all ethanol is produced in 8 h of process duration.

Table 3: Kinetic parameters of the alcoholic fermentation of enzymatically hydrolyzed liquor

Experiment	Yeast (g/L)	$(NH_4)SO_4$ (g/L)	KH_2PO_4 (g/L)	$Y_{p/s\ (Peak)}$ (g/g)	$Conv_{(Peak)}$ (%)	$Prod_{(Peak)}$ (g/L h)
1	5	0.3	0.05	0.317	0.621	0.381
2	15	0.3	0.05	0.348	0.680	1.253
3	5	0.9	0.05	0.349	0.684	0.840
4	15	0.9	0.05	0.386	0.756	1.393
5	5	0.3	0.19	0.427	0.835	2.052
6	15	0.3	0.19	0.383	0.750	0.921
7	5	0.9	0.19	0.422	0.825	2.028
8	15	0.9	0.19	0.453	0.887	1.635
9	10	0.6	0.12	0.460	0.901	1.660
10	10	0.6	0.12	0.382	0.747	1.377
11	10	0.6	0.12	0.353	0.692	1.275

Analyzing the effect of the three input variables studied on the influence of kinetic parameters, it could be concluded that to achieve better alcoholic fermentation efficiency, having as objective function the maximum % conversion of sugar into ethanol, the fermentation process must be operated at the central point of yeast concentration (10 g/L), nitrogen source (0.6 g/L) and phosphorus source (0.12 g/L), i.e., experiment 9, with % conversion 90 %. In the study of bioethanol production using acid hydrolysis in the raw material sisal (Lima et al., 2013) alcoholic fermentation efficiency of 92 % was obtained using industrial yeast *Saccharomyces cerevisiae*. More efficient acid hydrolysis was operated under the conditions of 3 % H_2SO_4 at temperature of 160°C in the bagasse: acid ratio of 1:10, obtaining maximum release of glucose in the liquor of 5.8 g/L. Compared with the present study, it appears that the efficiency (% conversion) was around 90 %, experiment 9, almost similar to the work carried out with sisal.

4. Conclusion

Evaluating the process of enzymatic conversion of cellulase into fermentable sugars, the enzymatic hydrolysis performance with 7 FPU and enzyme load of 3.5 FPU/g showed better efficiency for cashew bagasse in a time of 12 h. In operation conditions (concentrations of 10 g/L, 0.6 g/L and 0.12 g/L of yeast, source of N and P, respectively), ethanol was produced with 90 % conversion (optimal conversion of glucose to ethanol).
Cashew apple bagasse is a lignocellulosic material which showed a good performance in bioethanol production.

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