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Evaluation of Alkaline Delignification (Naoh) of Açaí Seeds (Eutherpe Oleracea) Treated with H_2so_4 Dilute and Effect on Enzymatic Hydrolysis

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Ethanol production from lignocellulosic residues from agriculture, such as the acaí seeds, have a fundamental step, delignification. This step becomes is more effective when performed in combination with others pre-treatments, preferably with those that are able to remove the hemicellulose fraction, such as treatment with dilute sulfuric acid. The aim this work was perform the delignification of açaí seeds pretreated with dilute sulfuric acid and evaluate the enzymatic hydrolysis rate. The açaí sees were pretreated with 1 % sulfuric acid, 10% solids for 60 minutes at 121 °C. An experimental set 2^2 for evaluate delignification was performed and the experimental conditions were 1 % NaOH, temperature from 39.64 to 110.36 °C (+α; - α) and solids loading from 3.79 to 46.21 % (-α; +α). After delignification the liquid fraction was characterized for carbohydrate content, lignin and fermentation inhibitors (acetic acid, furfural and HMF). And solid fraction was also characterized for the carbohydrate, lignin and acetic acid. Both fractions were characterized via HPLC. After delignification, the resulting material was enzymatically hydrolyzed with an enzyme loading of 15 FPU/g of seed and 25CBU/g of seeds and 3% of solids and glucose concentrations were measured via HPLC. The results showed that the yield varied from 24 to 49% and delignification was effective in removing up to 89 % of this pre-treated material with dilute sulfuric acid lignin. The condition of 7 5°C and 3.79 % of solids was the condition that promoted higher enzymatic conversion with a value of 94.76%. It can be concluded that the conditions for delignification studied were effective in removing lignin and improve glucose release during enzymatic hydrolysis.

1. Introduction

The replacement of fossil fuels for others less polluting is a strong trend of recent years. Following this technological aspect, is increasing the number of innovations, with different processes and new inputs used to produce these fuels. One of the greatest examples of possible substitutes for fossil fuels is the lignocellulosic ethanol. The importance of production of bioethanol from lignocellulosic feed stock has gained importance in the last few years due to various factors such as increased costs of petroleum fuel, benefits in environmental pollution control, energy security etc. (Sukumaran and Pandey, 2009).

The production of ethanol from lignocellulosic materials is considered a thriving technology, due to the large volume of material released and its low cost, especially when treating organic residues. The agro-industrial waste from food crops are an option for the production of fuels around the world. The use of waste for the production of ethanol, prevents competition between the production of raw materials for food or fuel production (Gómez et al., 2010).

In Brazil there are several studies on the use of residues of sucroalcooleira activity, such as bagasse and straw to produce ethanol. However some regions, such as northern Brazil, not have this raw material. But the processing of acaí releases every day in big cities of northern Brazil, as in the case of Belém and Manaus, a large amount of acaí seeds, material that could be used for the production of lignocellulosic ethanol. Amount of 70% of the fruit is discarded after its processing, in the form of seeds, without a more specialized use of this

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material. This material has been a sanitary inconvenience for these cities. The use of this material for fuel ethanol production purposes would be a way to add economic value for this material, and remove it from the public roads of these cities (Oliveira et al., 2014).
The efficient utilization of lignocellulosic material for ethanol production requires pre-treatments that can act in various forms on the lignocellulosic material, improving enzymatic hydrolysis and fermentation rates (Heredia-olea et al., 2013). The pretreatment step is applied in most cases to produce ethanol from lignocellulosic materials. In this step can be used as chemical agents as H_2SO_4 and NaOH, which act in different ways on the material and can be applied individually or combined (Resende et al., 2011). The acid pretreatment removes mainly the hemicellulose fraction of the material, while NaOH remove lignin fraction.
Treatment with NaOH can be applied in association with dilute H_2SO_4 to increase the efficiency of the pretreatment acid. The diluted solution of NaOH produced the various effects on the lignocellulosic material such as increasing the internal surface (swelling), decrease in the crystallinity and polymerization degree and structural separation of the linkages between lignin and carbohydrates, factors that improve the performance of hydrolysis (Pandey et al., 2011) . The pre-treatments must be analyzed specifically to each biomass used. Therefore this study aims to evaluate the alkaline delignification with NaOH pretreated acai seed with dilute sulfuric acid.

2. Material and methods

2.1. Material

Açaí seeds were collected in the city of Belém (01º27'21"S e 48º30'16"W) after of the process to obtained açaí juice. Then, the material was washed with water for removal of waste such as dirt and other unwanted components. The material was dried for 48 h at 50°C in an oven with air circulation and more 24 h at room temperature until moisture less than 10% moisture, condition recommended by NREL (National Renewable Energy Laboratory) for drying biomass purposes ethanol production. The material was milled (Willye model TE-650 - Tecnal – Brazil) to pass through of sieve of 9 mesh (2.0 mm) and retained in the sieve of 35 mesh (0.43 mm).

2.2. Açaí seeds pretreatments

Açaí seeds were initially hydrolyzed with diluted H_2SO_4 for (1% v/v in water) for 60 minutes at 120°C and pressure was kept at 1.05 bar and a 1:10 solid to liquid ratio (grams of açaí seeds/mL of solution) was used according Oliveira et al. (2014). The solid fraction was separated from the hydrolysate by filtration and abundantly washed with tap water to eliminate acid excess before oven drying at 30°C for 48 h. A second pretreatment step for açaí seeds was carried using NaOH solution (1% w/v) for 60 min. Was applied a set experimental 2^2 varying the load solids (3.79 – 46.25%) and temperature (39.64 -110.36°C) according table 1. Samples were then obtained by filtration, were washed until a neutral pH and dried in an oven for 24 hours at 30°C. After delignification, the solid and liquid fraction obtained after filtration was hydrolyzed and analyzed by HPLC and used for the enzymatic hydrolysis. The liquid fraction obtained after delignification was analyzed according to the method described by Kim et al. (1987) for all runs carried on experimental design. The set experimental applied is showed in table 1.

Table 1: Set experimental 2^2 applied for alkaline deslignification of açaí seeds from this work.

Run n°	Temperature	Load solids % (W/V)
1	50.00	10.00
2	50.00	40.00
3	100.00	10.00
4	100.00	40.00
5	39.64	25.00
6	110.36	25.00
7	75.00	3.79
8	75.00	46.21
9	75.00	25.00
10	75.00	25.00
11	75.00	25.00

2.3. Enzymatic hydrolysis

The cellulase (Sigma) activity was determined by the method described by Adney et al., (1996) and Ghose (1987) and the activity of β-glucosidase (Sigma) was determined according to the methodology of Wood 788 and Bhat (1988). Enzymatic hydrolysis of dried material was performed in Erlenmeyer flasks (250 mL) with 10 % (w/v) raw or pretreated material with sodium citrate buffer 0.05 M (pH 4.8) and 0.7 % of sodium azide/ g of dry material. The enzymatic loading used was 15 FPU/g dry material for Celluclast 1.5 (SigmaAldrich) and 25 CBU/mL material for β-glucosidase (Sigma-Aldrich) at 50 ºC, 150 rpm and 72 h. The enzymatic activity were 55 FPU/mL and 298 CBU/mL for celullase and β-glucosidase respectively.

2.4. Chemical composition

Approximately 8g of milled sample (only for untreated material) was extracted in two steps: first, with 190mL of ethanol 99% and in a second step with water using a Soxhlet tube and bags of semi-quantitative paper containing the samples. Ash content was determined after burning of the samples in a muffle at 550°C for 4 h (Ferraz et al., 2000) (for untreated and treated material). The untreated and pretreated seeds of açaí were analyzed for carbohydrate and lignin (acid-soluble and insoluble) according by Sluiter et al., (2008). Milled samples (0.3 g) were treated with 3 mL of 72 % (w/w) H_2SO_4, stirring for 1 h at 30 °C. The reaction was interrupted by adding 84 mL of distilled water, transferred to a 250 mL Schott flasks and then heated at 121°C/1 atm for 1 h. The residual material was cooled and filtered through porous glass filter number 3. The solids were dried until constant weight at 105°C and after calcinated in a muffle at 800°C for 2 hours and was determined as insoluble lignin. The soluble lignin concentration in the filtrate was determined by measuring absorbance at 205 nm and using the value of 105 L. $g^{-1}.cm^{-1}$ as absorptivity of soluble lignin. The hydrolysate was also analyzed by HPLC to determine carbohydrate, organic acid, furfural and hydroxymethylfurfural content. The released sugar monomers in the hydrolysate were determined by HPLC (Agilent) using a column (BioRad Aminex HPX-87H, 300x7.8 mm) at 35°C and 4mM H_2SO_4 as eluent at a flow rate of 0.6 mL min-1 injected sample volume 25 µm through of the detector RI (refractive index). The concentration of furfural and HMF were also measured following conditions: column Hewlett-Packard RP 18 (200 mm), Column temperature: 27°C UV detector SPD- 10A UV-VIS; eluent solution acetonitrile/water (1:8) with 1% acetic acid, injected sample volume of 20µm.

3. Results and Discussion

Table 1 shows the values of the chemical composition of acai seed untreated, pre-treated with diluted H_2SO_4 and acai seeds pre-treated with acid and delignification.

In Table 2, we can observe the results of the chemical characterization of the acai seeds in nature, after H_2SO_4 dilute pre-treatment and with subsequent delignification, which is considered for its calculation yields of both steps in material "in nature".

Thus, it can be seen that the yield ranged from 21.28 to 51.31%. The condition of run n°7 was that showed lower values hemicellulose (0.16 g/100 g of biomass untreated) and lignin (2.06 g/100 g biomass untreated) respectively, which indicates a greater removal these components for that condition, however for the same condition was also observed lower concentration of the cellulose (16.32%).

Oliveira (2010) studied deslignification of straw cane sugar pretreated with H_2SO_4 diluted with 10% solids loads and a solution of NaOH 1.5% at 190°C after 60 minutes, and achieved the maximum yield of 76%. This yield is similar however lower than the maximum yield was obtained for run n°8 from this study (46.21% solids at 75°C) it was 78% (calculated yield relative to the seeds pretreated with acid acai - not shown in table 2). The higher yields observed for the treatment of acai seeds can result from lower concentration of NaOH and temperature and higher concentrations of solids.

According to Table 2, it can be found that the cellulose yield was in a range of 16.32 to 32.53%, and the condition of the run n°8 (46.21% solids at 75°C) was that which allowed more maintaining the cellulose content after the delignification of açaí seeds treated with dilute H_2SO_4 material. The run n°7 (3.79% solids at 75°C) was the one that showed the greatest reduction (63.97%) of the cellulose content compared to untreated material.

Further in accordance with Table 2, when the solids content increased from 3.79% (Run 7) to 46.21% (Run 8) at 75°C temperature condition, an increase was observed in the remaining cellulose content of 99.32%. When solids were increased for 25% (Run 9, 10, 11) the increase on remaining cellulose content was of 88.90%. The increased delignification temperature of 75°C to 110°C at 25% solids resulted in a reduction in cellulose

content of about 10%. This behavior since maintenance is not desirable cellulosic fraction is of great interest for the later stages of the enzymatic hydrolysis and fermentation involved in ethanol production.
The condition of run n°7 (3.79% solids) showed lower lignin content (2.06 %) after delignification. The reduction in solids concentration of 40% (Run 2) to 10% (Run 1) at 50°C reduced the lignin content of approximately 46 %, while the increase in the temperature of 39.64 °C (Run n°5) to 110.36 °C (Run n°6) at 25% solids loading promoted a reduction of approximately 64 % in lignin yield. The levels of other components ranged from 0.07 to 0.16%, from 2.45 to 3.10 and 0.29 to 1.95% for the acetyl groups, ash and protein, respectively.

Table 2: Chemical composition of açaí seeds untreat, treated with H_2SO_4 dilute and treated with H_2SO_4 dilute and deslignificated

	Solids recovery	Run Cel[1]	Hem[2]	Lig[3]	AG[4]	Ash	Pro[5]	Total
Untreated	-	45.3	18.2	3.01	20.37	3.5	4.3	98.52
Treated with H_2SO_4	65.5	57.33	4.47	0.45	26.7	5.15	3.45	97.55
1	43.03	31.46± 0.29	0.65± 0.19	6.17± 0.33	0.15± 0.30	3.10± 0.15	1.67± 0.10	43.2
2	49.94	32.36± 0.25	1.90± 0.19	11.39 ±0.35	0.17± 0.29	2.70± 0.11	1.60± 0.13	50.12
3	39.82	31.34± 0.35	0.45± 0.21	4.30± 0.29	0.13± 0.21	2.99± 0.19	0.75± 0.22	39.96
4	46.18	31.86± 0.32	1.03± 0.21	8.40± 0.3	0.16± 0.20	3.09± 0.12	1.79± 0.19	46.33
5	45.59	30.64± 0.33	1.00± 0.2	9.25± 0.33	0.12± 0.22	2.92± 0.15	1.78± 0.11	45.71
6	34.37	27.53± 0.24	0.34± 0.17	3.37± 0.33	0.12± 0.23	2.78± 0.2	0.35± 0.15	34.49
7	21.28	16.32± 0.35	0.16± 0.17	2.06± 0.25	0.07± 0.23	2.45± 0.12	0.29± 0.15	21.35
8	51.31	32.53± 0.24	2.15± 0.23	11.65 ±0.23	0.12± 0.31	3.03± 0.1	1.95± 0.19	51.43
9	40.61	30.54± 0.31	0.85± 0.23	5.36± 0.31	0.13± 0.32	2.76± 0.15	1.10± 0.12	40.74
10	41.40	31.09± 0.24	0.79± 0.21	5.42± 0.32	0.13± 0.29	2.77± 0.11	1.32± 0.11	41.52
11	40.87	30.86± 0.25	0.82± 0.22	5.40± 0.27	0.13± 0.28	2.82± 0.13	0.98± 0.19	41.01

[1]-Cellulose; [2] – Hemicellulose; [3]-Lignin; [4]- Acetyl groups; [5]- Proteins

The chemical composition of liquid fraction obtained after delignification, in general, tends to be rich in lignin, which would be the main component of interest therein. Thus, liquid fraction after delignification was analysed only for lignin precipitated yelds. According to Figure 1 were found elevated amounts of lignin recovered from the liquid fraction, indicating a high efficiency of delignification step. The observed values ranged from 19.15 to 83.46%. The condition of run n°7 (3.79% solids, 1% NaOH at 75°C for 60 minutes) was recovered that demonstrated the highest lignin content, probably as a direct result of the efficient delignification of the solid fraction observed in this condition.

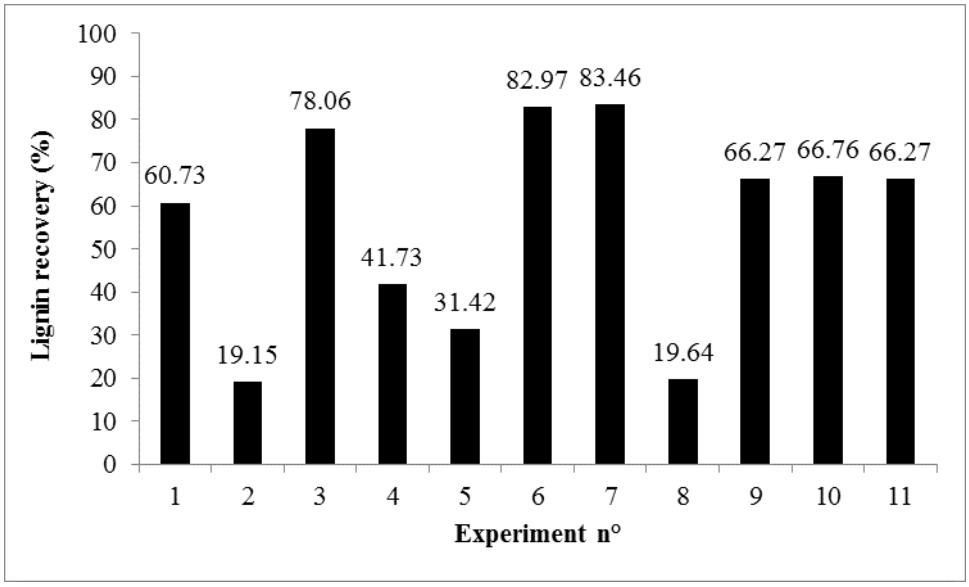

Figure 1. Precipitated lignin recovered in the liquor obtained after the delignification of açaí seeds treated with H2SO4 dilute.

In Table 3 are showed the results obtained from the enzymatic hydrolysis of biomass pretreated acai seed (in great condition considered) and delignified.

Table 3: Results obtained from the enzymatic hydrolysis of biomass pretreated acai seed (on optimal condition) and delignified

Run n°	Temperature (°C)	Load solids (%)	g glicose/g açaí seeds treated H_2SO_4 dilute	Conversion (%)	Global yield (%)
1	50	10	0.42	79.62	66.62
2	50	40	0.36	64.86	55.84
3	100	10	0.47	87.75	73.15
4	100	40	0.38	70.69	59.91
5	39.64	25	0.40	76.21	62.10
6	110.36	25	0.36	77.41	56.69
7	75	3.79	0.26	94.76	41.13
8	75	46.21	0.34	62.40	53.99
9	75	25	0.42	80.41	65.32
10	75	25	0.40	75.78	62.67
11	75	25	0.41	78.38	64.34

The values obtained for glucose concentrations ranged from 0.26 to 0.47 g glucose / g of seed pretreated with dilute acid. While the conversion of values ranged from 62.40 to 94.76%. The condition of run n°7 (3.79% solids at 75°C) was allowed to higher conversion. The optimum conversion value obtained for the material which was only pretreated with dilute acid was 66.48%. After delignification was possible to obtain a maximum increase up to a conversion of 42.53% in run n°7, which demonstrated the value mentioned conversion of 94.76%.

4. Conclusions

According working solids recovered after delignification acai prétradado core with H2SO4 ranged from 21.28% to 51.31% from 3.79% in terms of solids, 1 % NaOH at 75°C for 60 min (Run 7) and 46 21% solids, 1% NaOH at 75°C for 60 minutes (Run 8). The combination of pre-treatment with H_2SO_4 diluted with NaOH to alkaline delignification allowed maximum reduction of 99% in hemicellulose content. The low solids loads showed lower lignin content (2.6%) after delignification. The increased delignification temperature led to a reduction in

cellulose content and the condition which gave low hemicellulose content was once again test the condition of run n°7 (3.79% solids at 75°C) was that allowed greater enzymatic conversion of deslignificated açaí seeds. However, for this work was considered as optimum, condition from run n°9 (75 ° C and 25% solids) because optimal value 80.41%, obtained for enzymatic conversion, and the possibility of working with larger loads solids.

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