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# Effect of Particle Size on Dilute Acid Pretreatment and Enzymatic Hydrolysis of Sugarcane Bagasse

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Feedstock particle size can influence the lignocellulosic conversion yields and the energy costs of second generation ethanol production. In this work the influence of bagasse particle size on pretreatment and enzymatic hydrolysis was evaluated. Bagasse was ground using a knife mill equipped with screens to obtain particles of 0.5, 1.0 and 2.0 mm (sieve opening). Unmilled and milled samples were pretreated using dilute sulfuric acid in different conditions of temperature and time. The pretreated bagasses were subject to enzymatic hydrolysis for 72 hours. The pretreatment yield was determined from the mass loss during pretreatment for each assay and sample. The chemical compositions of untreated and pretreated bagasse were used to evaluate hemicellulose solubilization in pretreatment; and hydrolysis conversion was measured to assess the effect of particle size enzymatic saccharification. The higher mass loss and hemicellulose solubilization (39 and 87 %, respectively) occurred for the more severe conditions of pretreatment, 140 °C for 30 min. However, there was no statistically significant difference (p=0.05) of mass loss for the samples with different particle sizes. In enzymatic hydrolysis, the higher percentage of hydrolyzed mass was also for the pretreatment at 140 °C for 30 min (58-60), which was obtained >90 % of the sugars conversion. There was no significant difference (p=0.05) for hydrolyzed mass of samples with different particle sizes.

## 1. Introduction

In recent decades, the interest in the search for alternative energy sources to fossil fuel has grown considerably (Dias et al. 2012). The increasing energy demand results in a significant increase in fuel price (Lu et al. 2009), in addition to contributing to the greenhouse gas emissions from derived-oil combustion (Qi et al. 2010,). Lignocellulosic biomass is a potential feedstock for sustainable and economically viable production of bioethanol, due to its abundance and availability (Mood et al, 2013).
The sugarcane industry in Brazil has great importance in the economic context due to its efficient production of first generation ethanol and sugar (Palacios-Bereche et al. 2013), providing, as a byproduct of the process, large amounts of lignocellulosic biomass (Dias et al. 2013). Sugarcane bagasse (SB), as well as other lignocellulosic materials, is composed mainly of cellulose, hemicellulose and lignin (Takahashi et al. 2014). Cellulose is a homopolymer composed of glucose units linked by hydrogen bonds to form a crystalline structure of microfibrils. Hemicellulose is a polymer formed mainly by the pentoses xylose and arabinose. Lignin acts as a natural barrier to protect the cellulose and hemicellulose of biological degradation. The polymerized sugars can be hydrolyzed by enzymes to produce monosaccharides such as glucose and xylose, which are subsequently fermented by microorganisms to produce ethanol. However, the complex structure makes the enzymatic access in native material difficult (Benjamin et al. 2013).
Due to the complexity of plant structure, a pretreatment of the lignocellulosic material is essential to improve the saccharification of polysaccharides in fermentable sugars. The aim is to facilitate the enzyme

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access to cell wall carbohydrates, though without degradation of sugars and consequent formation of fermentation inhibitors compounds (Harrison et al. 2013).
Currently, the most studied pretreatments are the physical and chemical (or both). Physical pretreatments include different mills (particle size reduction) and irradiation. Chemicals pretreatments include acid, alkaline, solvent, supercritical fluid and oxidative pretreatments (Khullar et al. 2013). Physico-chemical pretreatments include steam explosion, ammonia fiber expansion (AFEX) and hot water (Mood et al. 2013).
Diluted acid is one of the more mature pretreatment methods (Chheda et al. 2007). When associated with moderate temperatures, it is effective in solubilization of hemicelluloses and recovery of sugars in the hydrolysate. Part of the cellulose may be converted into oligomers and monomers during the pretreatment, which may be advantageous as they are easily fermented by yeasts (Manzoor et al. 2012).
Particle size reduction is the first step to biomass conversion (Khullar et al., 2013). The increase of substrate surface area by size reduction facilitates the heat and mass transfer and enzyme accessibility, which could improve the efficiency of pretreatment and enzymatic hydrolysis (ZHU et al. 2009).
Vidal el al. (2011) reports that physical pretreatments alone resulted in efficiencies <50 % compared to chemical pretreatments that lead to >70 % conversion efficiencies. In addition, they show the influence of particle size depends on the pretreatment that will be used in the process. Therefore, optimizing biomass particle size is crucial to achieving high sugar conversion and low production cost (Zhu et al. 2009).
The objective of this study was to evaluate the effect of bagasse particle sizes in the dilute acid pretreatment and enzymatic hydrolysis. The effects of particle sizes on mass solubilization in different conditions of pretreatment and enzymatic hydrolysis were investigated.

## 2. Materials and methods

### 2.1 Raw material

Sugarcane bagasse, from a single harvest, donated by a sugar mill (Usina Tarumã do Grupo Raízen, Tarumã, São Paulo, Brazil), was used. The material was dried at room temperature and stored in plastic bags. A portion of bagasse was milled in different particle sizes (0.5, 1.0 and 2.0 mm) obtained by a knife mill equipped with a sieve (Fritsch - Pulverisette 19).

### 2.2 Pretreatment

Pretreatment was performed in laboratory scale stainless steel cylindrical reactors with a total volume of 500 mL. Bagasse samples (30 g of DM) were pretreated with 1 % (w/v) sulfuric acid at fixed solid-to-liquid ratio (1:5). The assays were performed at 120 °C for 20, 40, 60 min and 140 °C for 10, 20, 30 min. A glycerin bath was used to maintain the temperature. Initial time (t=0) was fixed as the time the desired temperature inside the reactor was reached. After the target pretreatment time was attained, the reactor was taken out from the glycerin bath and submerged into a water bath to cooling.
The wet pretreated material was squeezed to separate the liquid and solid fractions. The solid fraction was exhaustively washed with water and weighed subsequently. A portion of pretreated bagasse was used to chemical composition analysis and the rest of material was used to enzymatic hydrolysis.

### 2.3 Analysis of chemical composition

Untreated and pretreated bagasse were analyzed regarding chemical composition. Extractives content was analized according to Sluiter et al. (2005a) and ash content was analyzed according to Sluiter et al. (2005b). Structural carbohydrates and lignin were analyzed according to Sluiter et al. (2008) and adapted by Gouveia et al. (2009).
Sugars, acetic acid, HMF and furfural were analyzed by HPLC (Agilent Technologies). Cellobiose, glucose, xylose, arabinose and acetic acid were separated on an Aminex HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 35 °C and 0.05 mM $H_2SO_4$ as eluent at a flow rate of 0.6 mL/min using a refractive index (RI) detector. HMF and furfural were separated on Nova-Pak C18 column (Waters Co., Milford, MA) at 30 °C and a solution composed of a 1 % acetic acid-containing 1:8 acetonitrile-water at flow rate of 0.8 mL/min was used as eluent.

### 2.4 Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated bagasse was performed in erlenmeyers with a substrate content of 10 % (DM) and sodium citrate buffer 0.05 mol/L at pH 4.8. The erlenmeyers were incubated in an orbital shaker MA-832 (Marconi, Piracicaba, SP, Brazil) agitated at 150 rpm at 50 °C for 72 h.
Cellulase (Celluclast 1.5 L (80 FPU/g)) and β-glucosidase (Novozym 188 (616 CBU/g)) were used. The enzyme loadings used were 15 FPU/g bagasse of cellulose and 25 CBU/g bagasse of β-glucosidase.

The hydrolyzed sugars were determined by HPLC on an Aminex HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 35 °C and 0.05 mM $H_2SO_4$ as eluent at a flow rate of 0.6 mL/min using a refractive index (RI) detector.

## 3. Results and discussion

### 3.1 Chemical composition of untreated bagasse

The chemical compositions of untreated bagasse in different particle sizes are presented in Table 1.

*Table 1: Chemical compositions of untreated bagasse in different particle sizes*

| Sample | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Ash (%) | Extractives (%) |
|---|---|---|---|---|---|
| Unmilled | 40.35 ± 0.21 | 28.12 ± 0.00 | 25.79 ± 0.26 | 2.35 ± 0.18 | 2.98 ± 0.11 |
| <0,5 mm | 38.12 ± 0.11 | 26.93 ± 0.26 | 26.70 ± 0.15 | 4.10 ± 0.22 | 4.12 ± 0.04 |
| <1,0 mm | 40.02 ± 0.11 | 27.76 ± 0.07 | 25.66 ± 0.35 | 2.37 ± 0.12 | 3.67 ± 0.01 |
| <2,0 mm | 39.63 ± 0.08 | 27.90 ± 0.09 | 26.44 ± 0.19 | 2.54 ± 0.48 | 3.30 ± 0.01 |

From Table 1 it can be seen that bagasse with smaller particles (<0.5 mm) presents a slightly lower cellulose percentage.

### 3.2 Effect of particle size on pretreatment

Pretreatment assays in different conditions of time and temperature are presented in Table 2.

*Table 2: Pretreatment assays*

| Assay | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 120 | 0 |
| 2 | 120 | 20 |
| 3 | 120 | 40 |
| 4 | 120 | 60 |
| 5 | 140 | 0 |
| 6 | 140 | 10 |
| 7 | 140 | 20 |
| 8 | 140 | 30 |

Time zero was defined as the time necessary for the inside of the reactor to reach the desired temperature.

Figure 1 shows the solubilized mass (% DM) in the different samples during the pretreatment.

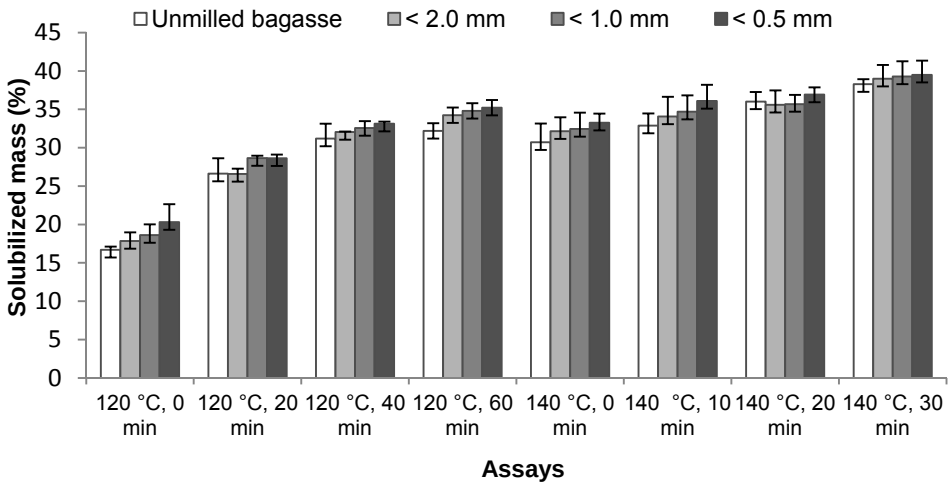


*Figure 1: Solubilized mass (% DM) of bagasse samples with different particle sizes.*

It can be noted from Figure 1 that the solubilized mass was in the range from 17 to 39 %. It is observed in all assays that bagasse with smaller particles (<0.5 mm) presents a slightly higher solubilization compared to the other particle sizes. Tukey HSD test was performed to evaluate if the difference between the samples was statistically significant. The software used was STATISTICA 7.0 and the results at 95 % of confidence showed that the difference among solubilized mass (%) for the samples with different particle sizes submitted to the same pretreatment conditions was not significant.
Chung et al. (2012) reports that mass loss in dilute acid pretreatment is due the hemicellulose solubilization present in the biomass. Figure 2 shows the hemicellulose solubilization (% DM) in the pretreatment.

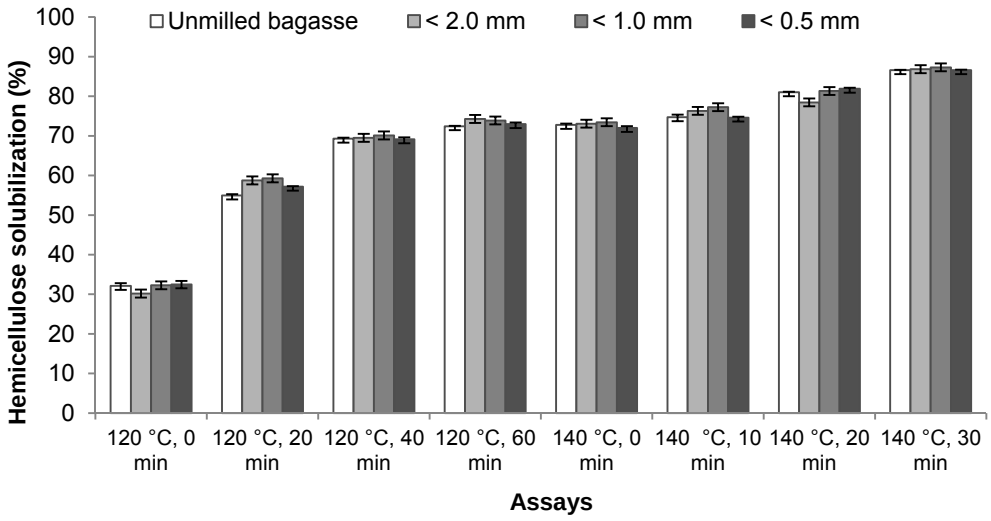


*Figure 2: Solubilized hemicellulose (% DM) in the pretreatment.*

Figure 2 shows that the range of solubilized hemicellulose was from 30 to 87 %. The higher solubilization (87 % of hemicellulose) occurred in the more severe condition, 140 °C for 30 min. As expected, the lower solubilization occurred in the less severe assay (120 °C, 0 min), and varied from 30 to 33 % between different samples.
It can also be noticed from Figure 2 that the assays at 120 °C, 60 min had hemicellulose solubilizations similar to the assays at 140 °C, 10 min (72-74 %). Thus, the pretreatment was influenced by temperature, since the reaction at 140 °C solubilized more quickly the same amount of hemicellulose when compared to the assay performed at 120 °C.
Regarding particle size it was observed that the different sizes did not influence solubilization, since the amount of solubilized hemicellulose in the samples with different particle sizes submitted to the same pretreatment conditions was statistically equal (p=0.05).
Kim and Lee (2002) proposed a model to evaluate the intraparticle diffusion of sulfuric acid in different feedstocks and the effect on dilute sulfuric acid pretreatment. Vidal et al. (2011) used this model to calculate the theoretical critical particle size (the size above which diffusion becomes important in the pretreatment process) for different feedstocks (corn stover, bagasse, hardwood, wheat straw) pretreated with dilute sulfuric acid (0.5 % w/w) at 180 °C. The values were in the range of 1.0 to 3.0 mm (sieve opening) suggesting that smaller particle sizes do not increase the pretreatment efficiency. The results of the present work, indicates that for the pretreatment conditions considered, even unmilled bagasse leads to similar pretreatment performance.

### 3.3 Effect of particle size on enzymatic hydrolysis

Figure 3 shows the hydrolyzed mass of untreated and pretreated samples. It can be noted clearly the difference of solubilization in the untreated samples (13-18 %) compared to pretreated samples (33-60 %), evidencing the increase of enzymatic hydrolysis efficiency after pretreatment. The higher mass loss (58-60 %) occurred in the samples pretreated at 140 °C for 30 min (assay 8), in which the higher hemicellulose solubilization in pretreatment was also observed (Figure 2).

Regarding particle size, a solubilization about 5 % higher occurred for the milled samples compared to unmilled bagasse; however, a Tukey HSD test showed no significant difference among samples (p=0.05), indicating no influence of particle size on enzymatic hydrolysis, the same behavior observed in pretreatment.

Hsu et al. (1996) observed an increase in the digestibility of switchgrass from 60 to 80 % when the particle size decreased from 10 to 3.0 mm. However, no difference was observed when pretreated materials of different particle sizes were homogenized and subjected to enzymatic hydrolysis, indicating that particle size has no influence on the performance of pretreatment. Khullar et al. (2013) did not observe difference in the glucose release rate of enzymatic hydrolysis of *Miscanthus* with particles of 0.08 and 2 mm (sieve opening) pretreated with diluted acid.

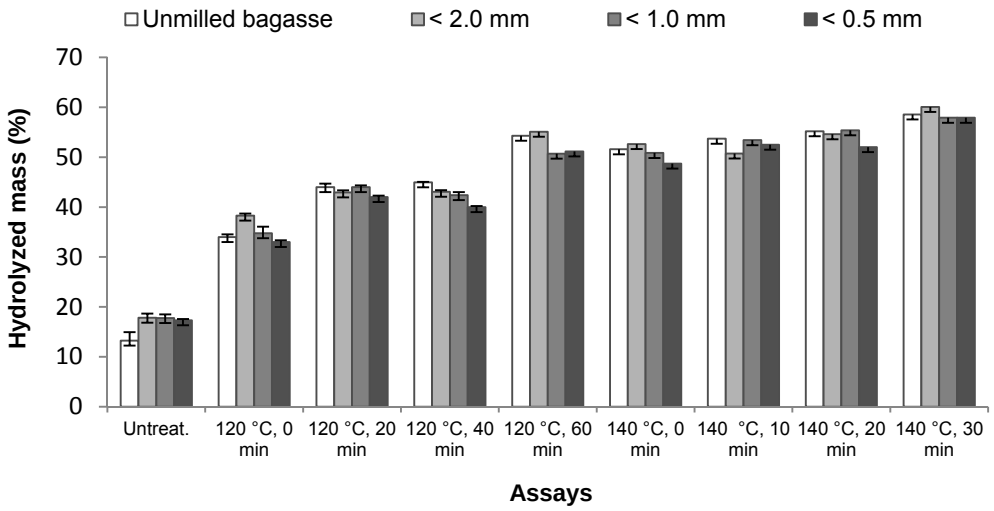


*Figure 3: Hydrolyzed mass (% DM) of pretreated samples.*

The chemical composition and the hydrolyzed mass of the samples pretreated at 140 °C for 30 min (assay 8) are shown in Table 3.

*Table 3: Chemical composition and hydrolyzed mass of pretreated bagasse samples at 140 °C for 30 min*

| Sample | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Ash (%) | Hydrolyzed mass (%) |
|---|---|---|---|---|---|
| Unmilled | 54.84 ± 0.22 | 6.16 ± 0.05 | 33.71 ± 0.42 | 5.36 ± 0.08 | 58.55 ± 0.10 |
| <0,5 mm | 55.60 ± 0.57 | 6.09 ± 0.01 | 34.38 ± 0.42 | 4.83 ± 0.13 | 57.89 ± 0.31 |
| <1,0 mm | 55.20 ± 0.16 | 5.94 ± 0.02 | 33.50 ± 1.01 | 4,19 ± 0.05 | 57.88 ± 0.61 |
| <2,0 mm | 56.04 ± 0.27 | 6.15 ± 0.11 | 33.65 ± 0.35 | 3.64 ± 0.01 | 60.06 ± 0.20 |

Considering the percentages of the sugars in the pretreated materials and the percentage of hydrolyzed mass it seems that high hydrolysis conversions were attained (>90 %), since the percentage of hydrolyzed mass is higher than the percentage of cellulose in the pretreated materials. The commercial enzyme Celluclast contains high activity of acetil xilan esterases (Juhász et al. 2005), thus hemicelluloses are also hydrolyzed.

## 4. Conclusions

No difference was observed among samples with different particle sizes in the pre-treatment and enzymatic hydrolysis, indicating that the range of particle sizes studied did not influence these steps. The pre-treatment at 140 °C removed most of the hemicelluloses in reaction times shorter than the pretreatment at 120 °C. The higher percentage of solubilized mass in the pretreatment (38-39 %) and enzymatic hydrolysis (58-60 %) occurred at 140 °C, 30 min.

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