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Enzymatic Hydrolysis Exploration and Fermentation: Acid Pretreatment and Delignification in Sugarcane Bagasse for 2G Ethanol Production

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Renewable energy is described in the political sphere as a means to reduce greenhouse gas emissions and simultaneously increase energy security, especially in the transport sector, which is dependent on oil products. Enzymatic hydrolysis is an intermediate step in the biomass conversion process into fermentable sugars. This step has to be favorable to produce high sugar yields and the resulting hydrolysate must be able to support fermentative organisms while they produce biofuels.
In this investigation sugarcane bagasse was submitted to a chemical pretreatment with dilute sulfuric acid (121 °C, 80 min and 1.0 % v/v H_2SO_4) and then delignification with alkaline solution (80, 100 and 120 °C, 30, 60 and 90 min, 0.5, 1.0 and 1.5 % w/v NaOH) both with 20 % w/v of solids loading. The samples were submitted to enzymatic hydrolysis with 8.0% w/v solids loading (dry basis) considering 15 FPU (filter paper unit) and 33.0 CBU (beta-glucosidase unit) per gram of dry biomass. The fermentation was applied on the 0.5 % w/v NaOH, 80 °C, 90 min delignificated point.
The enzymatic hydrolysis process favored the release of fermentable sugars, given the fact the values for the concentration of total reducing sugars increases with hydrolysis time. However, it is shown that after 24 h of hydrolysis occurs the maximum liberation of sugars. Results from fermentation process showed that $Y_{P/GLC}$ for pretreated sugarcane bagasse was about 0.51 kg_{etanol}/kg_{GLC} and for the delignificated was 0.47 kg_{etanol}/kg_{GLC}. It was attained a process yield of 100 % for the pretreated sugarcane bagasse and 91.59 % for the delignificated.

1. Introduction

Research on climate change and energy security, as well as on the interactions between them, require the development of routes that make use of renewable lignocellulosic material. Renewable energy is described in the political sphere to reduce greenhouse gas emissions and simultaneously increase energy security, especially in the transport sector, which is dependent on oil products (Mansoon et al., 2014). Besides the use of renewable feedstock to produce biofuel may a good strategy to improve the agribusiness.
For the large-scale biological production of ethanol, it is desirable to use cheaper and more abundant substrates and consequently reduce their price, with a positive impact on the market to use of ethanol as fuel and intermediate for chemicals. Lignocellulosic biomass is considered an attractive feedstock for the production of ethanol, due to its availability in large quantities and relatively low cost. This is typically the cane of sugarcane bagasse that is a byproduct from the first generation process and readily available at the production site.
The process for obtaining ethanol from lignocellulosic biomass requires some steps, with different possible combinations and operations: enzyme production, pretreatment, enzymatic hydrolysis and fermentation.
Contemplating the evaluation of different operational scenarios of pretreatment processes, hydrolysis, and fermentation in the production of 2G ethanol, sugarcane bagasse must undergo a pretreatment step to

transform the bagasse, with advantages in the following acid or enzymatic hydrolysis step. Since lignocellulosic materials have a complex and compact structure, the pretreatment step is necessary for disruption of the cellulose-hemicellulose-lignin complex. This is one of the most expensive steps in the process of converting biomass into sugars.

In this context, the chemical delignification process includes all processes that result in partial or complete removal of lignin by the action of suitable reagents. In addition to the occurrence of purely chemical events, these processes comprise a number of morphological and physical phenomena. The delignification is carried out by two types of structural changes in the lignin that can be connected to each other. The first involves the degradation by cleavage of certain interunit connections, and the second involves the introduction of hydrophilic groups in the polymer and its fragments (Gierer, 1985).

In 2G ethanol processes, the hydrolysis involves breaking the glycosidic bonds of polysaccharides, which are the raw material pretreated in fermentable sugars (i.e. hemicelluloses and cellulose, which consist mainly of C5 and C6 sugars, respectively). Monosaccharides provide a water molecule to inactivate the broken connection (Wettstein et al., 2012). The methods most frequently applied for the hydrolysis are the chemical hydrolysis (acid hydrolysis) and the enzymatic hydrolysis.

Enzymatic hydrolysis is an intermediate step in the conversion of biomass to fermentable sugars yielding, at the same time, high yields of sugar. Hence, the resulting hydrolysate must be capable of withstanding subsequent fermentation organisms as they produce biofuels. The main factors influencing the enzymatic hydrolysis of cellulose from lignocellulosic raw materials can be divided into two groups: factors related to the enzyme and factors related to the substrate, although many of them are interrelated during the hydrolysis process (Alvira et al., 2010). This work describes the enzymatic hydrolysis for sugarcane bagasse with acid pretreatment (AP) and then by alkali delignification (DLG) in a variety of conditions. In sequence, the best delignification point is submitted to enzymatic hydrolysis (EH) and fermentation for 2G ethanol production.

2. Material and methods

Sugarcane bagasse (SCB) was donated by a Brazilian sugar-alcohol-co-generation mill (Usina São João, Araras, São Paulo - Brazil) and the dry matter (DM) composition is presented in Figure 1.

Acid pretreatment (AP), alkali delignification (DLG) and enzymatic hydrolysis (EH) were carried out as exhibited in Figure 1, for the following experimental conditions: AP - 1 % w/v H_2SO_4,121 °C, 80 min and 20 % w/v solids loading; DLG - 0.5, 1.0, 1.5 % w/v NaOH, 80, 100, 120 °C, 30, 60, 90 min and 20 % w/v solids loading; EH - 50 °C, 72 h, 150 rpm, 15 FPU/g and 33 CBU/g substrate, 8 % w/v solids loading. Insoluble solids fractions (ISF) were analyzed based on standard procedures (Canilha et al., 2011, Gouveia et al., 2009, Sluiter et al., 2008).

The values presented for DLG in Figure 1 correspond to the point (0.5 % w/v NaOH, 80 °C and 90 min). These were the most favourable delignification conditions with 88.98 ± 0.63 % of process yield and 75.41 ± 0.73 % of lignin removal.

The experimental development of the fermentation process was considering the AP and the AP + DLG (in the point previously mentioned) subsequently subjected to enzymatic hydrolysis. The reaction volume consisted of 75 % v/v hydrolysate and 25 % molasses solution (Herrera et al., 2016).

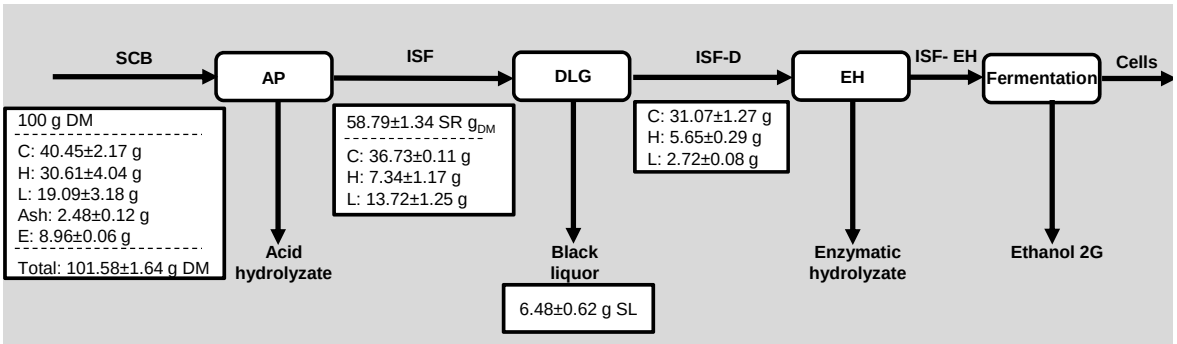

Figure 1: Schematic representation of the developed process.C: Cellulose; H: Hemicelluloses; L: Lignin; E: Extractives; SR: Solid Recovered; SL: Soluble Lignin; ISF: Insoluble Solid Fraction; ISF-D: Insoluble Solid Fraction-Delignification; ISF-EH: Insoluble Solid Fraction-Enzymatic Hydrolysis.

3. Results and discussions

3.1 Enzymatic Hydrolysis

In Figure 2 is depicted the evolution of total reducing sugars (TRS) concentration and glucose (GLC) during the enzymatic process for all the delignification points.

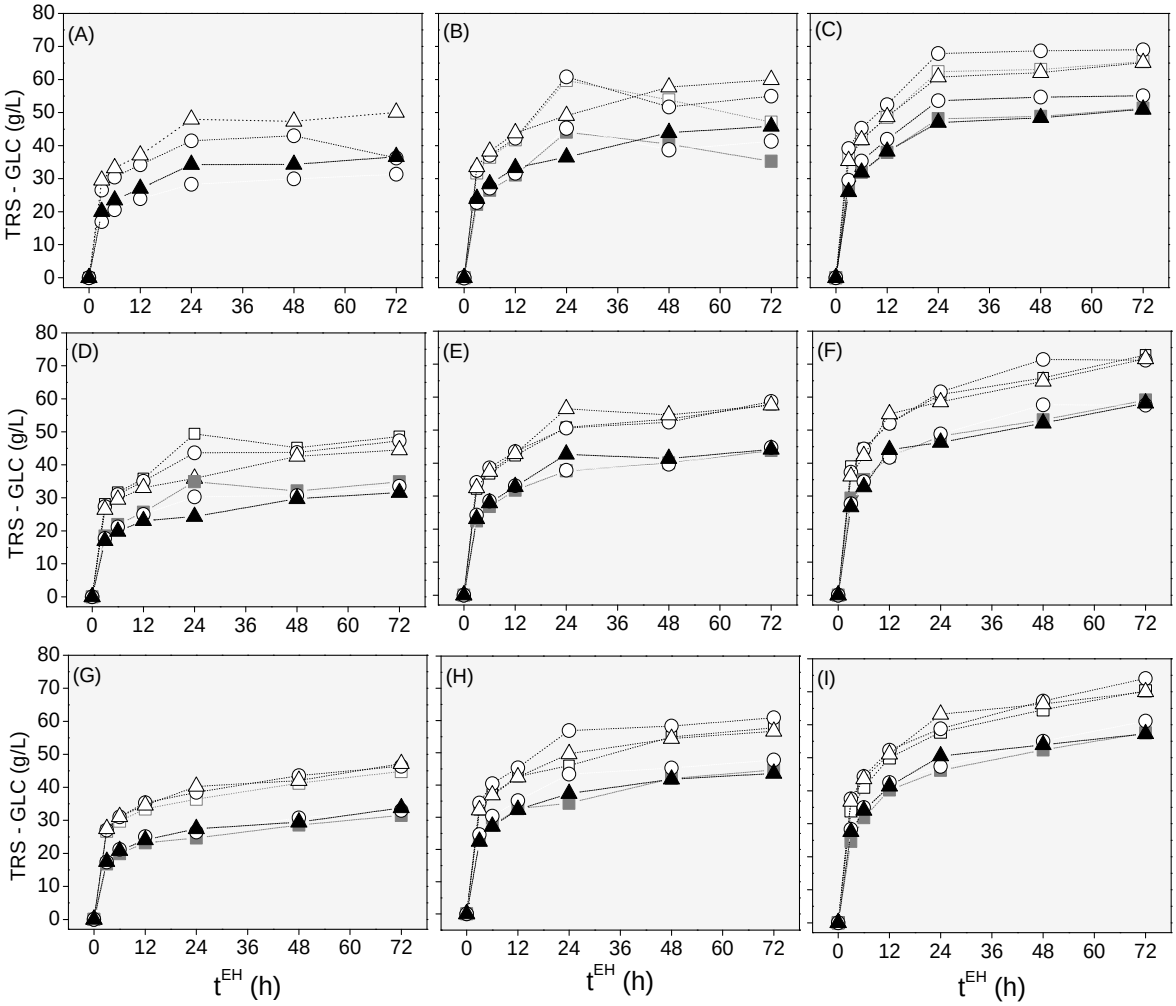

Figure 2: Values of total reducing sugars (TRS (g/L) – open symbols) and glucose concentration (GLC (g/L) – closed symbols) as a function of enzymatic hydrolysis time (t^{EH}). Delignification condition: Temperature: 80 °C (A, B, C), 100 ºC (D, E, F), 120 ºC (G, H, I); NaOH: 0.5 % w/v (A, D, G) , 1.0 % w/v (B, E,H), 1.5 % w/v (C, F, I); Time: □/■ 30 min ○/● 60 min △/▲ 90 min.

By analyzing the graphs, it is possible to note the same behavior: values increase until 24 h enzymatic hydrolysis elapsed and then become almost constant until the end of the process. Exceptions occur in Figure 2.B for 30 and 60 min that present a decrease in the sugars concentrations due to inhibitory enzyme processes, and in Figure 2.F and 2.I, where the values continue to increase after 24 h although with a lower rate.

Comparing all curves together, it is noted that with increasing the NaOH concentration from 0.5 to1.5 % w/v, there is a significant increase in TRS and GLC concentrations. They vary from approximately 50 g/L with 0.5 % w/v to 70 g/L with 1.5 % w/v.

It is also noted that in Figure 2-A, the highest TRS values are reported in 90 min residence time. In Figure 2-B, the values behave in a disorderly way, but the values in 90 min are greater than the others. Already in Figure 2-C, the higher reported amounts are with 60 min.

In Figures 2-D, 2-E and 2-F, when is considered the equivalent reaction time of the delignification process, the values are very close to each other. The same occurs for Figure 2-G, 2-H and 2-I. Some differences occur at

specific points, i.e., in Figure 2-D, at 24 h of enzymatic hydrolysis, the value for 30 min are higher than the others and achieved approximately 50 g/L.
The increase in TRS values from 24 to 48 and 72 h steps are small. They range from 12 g/L (100 °C, 1.5 % w/v NaOH and 30 min point) to non-existent in some cases (e.g. at 80 °C, 1 % w/v NaOH and 30 min point). In this way, it appears that 24 h would be enough time for the effective sugars release from the enzymatic hydrolysis, carrying the maximum sugars conversion.
Enzymatic hydrolysis were carried out in the raw sugarcane bagasse and in the ISF. The results revealed that the first experiment had a glucose release of 1.23 g/L with a glucose yield of 3.32 %. Already for the second case it was obtained a glucose release of 34.54 g/L with a yield of 62.27 %. This means that AP and delignification provide a satisfactory effect on glucose release during the enzymatic hydrolysis.
Gao et al. (2013) conducted different combinations of sugarcane bagasse pretreatment, namely superheated water (LHW) and sodium hydroxide (1 % w/v NaOH). The enzymatic hydrolysis was carried out in 5 % solids loading at 50 °C, 150 rpm and enzyme loads of 5, 10, 20, 30, 40, 50 FPU/g glucan. With 30 FPU/g glucan after 72 h of hydrolysis about 37 g/L of glucose was obtained for the LHW+NaOH sequence and 38 g/L for the inverse sequence (i.e. NaOH+LHW). These very close values indicate the order of pretreatments is irrelevant. Considering the difference in pretreatment and enzymatic hydrolysis conditions applied the values reported by the author are consistent to those reported in this work.
The sugarcane bagasse assessed by Rocha et al. (2013), was pretreated hydrothermally with 10 % solids loading in different temperature ranges and then delignificated with 1.0 % w/v NaOH for 1 h at 100 °C. The enzymatic hydrolysis was conducted using 10 IU and 15 FPU, 100 rpm, 45 °C for 72 h and the values obtained for glucose concentration were about 63.1 ± 0.5 g/L for the pretreatment at 180 °C and 71.6 ± 0.8 g/L at 185 °C. These values are close to those found in this work for the delignified samples at 120 °C, proving the efficiency of the high temperature pretreatment applied in the glucose release.
The global yields have increased their values with hydrolysis time. For example, in 0.5 % w/v NaOH, 90 min and 80 °C reach 29.21 % with 3 h of hydrolysis, about 50.04 % with 24 h reaching 53.47 % at 72 h.
With the temperatures applied in DLG the best values achieved for the global yield is with 1.5 % w/v NaOH. At 80 °C and 60 min was 76.83 %, at 100 °C and 30 min this value is about 64.54 % and at 120 °C and 60 min was 64.22 %.
Regarding the NaOH concentration applied, the obtained values increase with the rise of the applied concentration. The lower values are found in 0.5 % w/v and the bigger in 1.5 % w/v NaOH.
The process time showed no orderly behaviour in relation to yield values.

3.2 Fermentation Process

Figure 3 shows the profiles obtained during fermentation of pretreated SCB and of pretreated SCB followed by alkali delignification in the chosen point.
After culture medium inoculation is observed that the adapting process of the yeast in the enzymatic hydrolysate matrix is favorable, since the TRS data concentration decreases over the hours course, thus demonstrating the performance achieved by *S. cerevisiae* with respect to consumption of the substrate (Figure 3).
In the first hours no ethanol formation (0 – 2 h) is observed due to the lag phase. This corresponds to an adaptation period, where the cell synthesizes enzymes necessary for metabolism of the medium components. This phase is not very extensive due to the inoculum process, cells preculture in culture medium with hydrolysate and nutrient salts. This benefits the reduction of this step and the beginning of the transition stage which describes the increase in cell formation rate with an exponential increase of the quantity of biomass.
Subsequently, the process presents a linear increase where the velocity rate is constant, followed by a deceleration due to the exhaustion of one or more culture medium components necessary for growth, and also the accumulation of inhibitory metabolites. At that time, growth and specific rates decrease until annul in end time (t_f).The stationary phase in sequence describes a maximum cell concentration reached equivalent to 8.03 g/L (at 40.75 h) and 11.3 g/L (in 36 h) for the fermentation process of the AP and that followed by delignification, respectively.
Therefore, upon reaching stationary phase there is a balance between the rate of formation and microorganism cell death, with chemical changes occurring within the cell. During the fermentation process, it is observed glycerol production (Figure 3) which is the largest by-product of the alcoholic fermentation by *S. cerevisiae* performed, typically 2-3 % fermented sugar compound that is converted (Jain et al., 2011). The amount of glycerol reached after fermentation for the AP and the followed by delignification is 7.32 g/L (at 40.75 h) and 5.91 g/L (in 36 h), respectively.

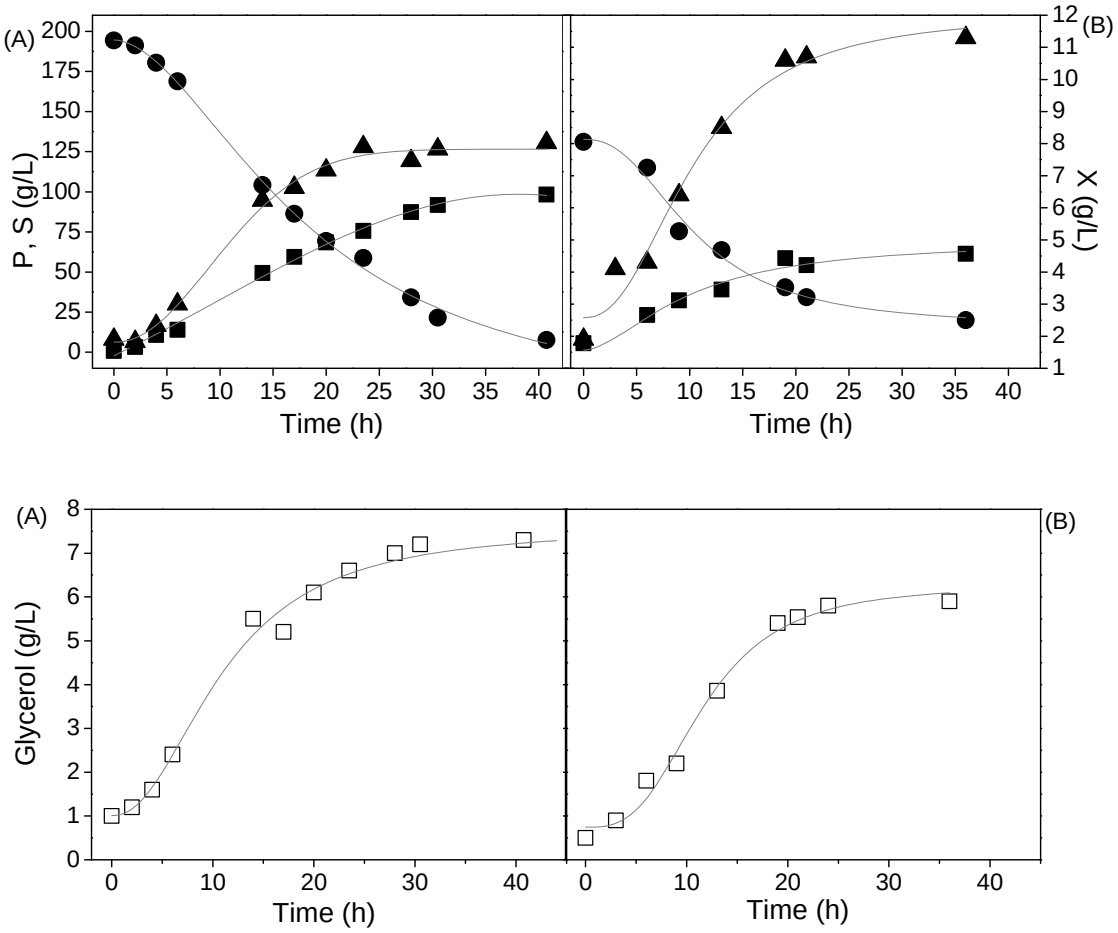

Figure 3: Concentration profiles during the fermentation of pretreated SCB under optimum conditions (A) and for delignificated SCB (B): substrate concentration, S (●); ethanol, P (■), total cell, X (▲) and glycerol, G (■).

Productivity represents the variation of the ethanol formation in relation to time. For the AP was obtained around 2.71 g/Lh (in 40.75 h) of productivity and for AP+DLG this value was 1.55 g/Lh (in 36 h).

The conversion factors $Y_{X/GLC}$ and $Y_{P/GLC}$ are magnitudes that relate, at a given time *t,* the corresponding values of X, S and P, allowing to quantify the transformation of the substrate into cells and substrate in ethanol.

The $Y_{P/GLC}$ value at the end time (t_f) relates the final ethanol amounts produced when all initial sugars were fermented. In the fermentation process of AP and followed by delignification, these parameters reach 0.51 $kg_{ethanol}/kg_{GLC}$ and 0.47 $kg_{ethanol}/kg_{GLC}$ respectively. $Y_{P/GLC}$ values in a range between 0.408 kg/kg to 0.465 kg/kg were reported by De Andrade et al. (2013), where sugarcane bagasse has been pretreated with 4 % of dry matter and hydrogen peroxide solution, subjected to enzymatic hydrolysis with a 3.0 % (w/w) concentration of substrate, 3.5 FPU/g and 25 CBU/g dry matter, at 100 rpm and 50 °C.

The last parameter is the efficiency, establishing a relationship between the $Y_{P/GLC}$ of enzymatic hydrolysate fermentation and the theoretical $Y_{P/GLC}$, which would be the maximum possible grams conversion of glucose to ethanol (0.511 $kg_{ethanol}/kg_{GLC}$). The of the fermentation process global yield for the AP and for the process with delignification were 100 % and 91.59 %, respectively. Tan and Lee (2014) used solid residue obtained after extracting algae from k-carrageenan, which reached for the fermentation process, a yield of 90.9 % ethanol.

The fact that a yield of 100 % for the fermentation step in pretreated material is due to the operation conditions applied. This step was carried out at CTBE (Brazilian Bioethanol Science and Technology) in a suitable reactor, with specialized equipment in the procedure.

4. Conclusions

The experimental data obtained in this work and their evaluation reach the conclusion that maximum sugars liberation occurs with 24 h of enzymatic hydrolysis pretreatment. There were some little improvements, with an increase at 48 and 72 h. The reported process AP + DLG + EH had satisfactory performance, since it provides a total reducing sugars release from 69.98 g/L to 74.05 g/L, and enzymatic hydrolysis global yield of 65.40 % to 71.49 %.

Regarding the fermentation, the hydrolysates led to ethanol production. However, further in-depth investigation is required to optimize the whole process, from pretreatment to fermentation.

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