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Bioethanol from Brewer's Spent Grains: Acid Pretreatment Optimization

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This study performs a parametric study aiming at the optimization of the acid pretreatment step of brewer's spent grains (BSG) simultaneously with the enzymatic hydrolysis for conversion into simple sugars fermentable to bioethanol. For this purpose three acids and five enzymes were tested, by adding each two acids (HCl with H_2SO_4 or HCl with HNO_3) either in mixture (in one step) or sequentially (in two steps), to 25 g of dry BSG, together with varying quantities of the enzymes. Results show that when using Viscozyme L or the mixture of Cellulase and Hemicellulase by action of two acids in mixture, the total sugars conversion ranges between 20-27 wt%, in which the mixture of HCl and H_2SO_4 promotes a greater release of glucose plus maltose, while the mixture of HCl and HNO_3 promotes the release of higher amount of xylose and arabinose. Results also show that when Glucanex 100g and Ultraflo L are used simultaneously with the sequential addition of HCl and H_2SO_4, the highest total sugars conversion (54.5 wt%) is obtained using 2.30 mL of Ultraflo L and 1.67 g of Glucanex 100g. Furthermore, by increasing the amount of Glucanex 100g (from 1.67 to 2.48 g) to the same amount of Ultraflo L (2.30 mL) the total sugars conversion decreased from 54.5 wt% to 40.5 wt%. Moreover, a greater release of glucose was verified by increasing the amount of Ultraflo L (from 1.75 mL to 2.30 mL), while by increasing the amount of Glucanex 100g (from 1.67 to 2.48 g) the release of arabinose and maltose was enhanced. Also, when using Glucanex 100g and Ultraflo L simultaneously with the acids HCl and HNO_3, the best method to obtain high conversions of sugars is by the sequential addition of the acids, instead of in mixture. In this work, it resulted in the best conversion of BSG to simple sugars (72.1 wt%), corresponding to about 720 g of sugars per kg of dry BSG.

1. Introduction

The development and production of alternative fuels, such as bioethanol, is seen as a viable option to reduce our dependence on fossil fuels and help alleviate their impact on the environment by reducing the greenhouse gas (GHG) emissions and thus, the global warming (Mata et al., 2010). Bioethanol can be used to replace conventional gasoline in today cars with little or none modifications of vehicle engines. It can be produced through physical processes (gasification) or bio-chemical processes (enzymatic hydrolysis and fermentation) from organic materials (e.g. sugarcane, sweet sorghum, wheat, grit corn, potato, wood, and grasses) and residues (e.g. molasses, straw, bagasse, pulp, and waste sawdust) (Mata et al, 2011). The compositions of these materials vary, being the major component cellulose (35 – 50 %), followed by hemicellulose (20 – 35 %) and lignin (10 – 25 %) (Saha, 2003).
The industrial production of bioethanol is predominantly from agricultural crops such as maize (in USA) and sugarcane (in Brazil), leading to a direct conflict with food and increasing usage of pesticides and herbicides in agriculture practices, thus contributing to social and environmental impacts. For these reasons the increasing demand for alternative fuels, has to be satisfied with other biomass sources, such

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as the lignocellulosic materials, considered to be second generation biofuels feedstocks (White et al., 2008). Hence, this work identified the brewer's spent grains (BSG) as a rich source of lignocellulose (Jay et al., 2008) that can be converted into fermentable sugars for bioethanol production (Mussatto et al., 2006).
The main by-product in the brewing industry is the BSG, a lignocellulosic material consisting of the husk and outer shell of the cereals that remain after the mashing process that represents about 85 % of the total by-products of this industry (Aliyu and Bala, 2011). This waste material is rich in cellulose and hemicelluloses that can be converted into simple sugars and those fermented into ethanol (White et al., 2008). On a dry weight basis, BSG contains about 40 - 50 % polysaccharides (consisting of 15 - 18 % cellulose, 24 - 31 % hemicellulose and 2 - 3 % starch) and 30 % or more proteins (Macheiner et al., 2003). As a residue of mashing, it contains about 80 % of water, thus preventing long-term storage due to rapid microbial spoilage. The brewing industry has been developing much effort to reduce the processes energy demand. An example of this is the “Green Brewery” project in a joint cooperation between industry and research, aiming to identify possible solutions to reduce the fossil CO_2 emissions in the thermal energy generation for breweries. In this project it was concluded that using BSG as raw material for combustion could be interesting, if the water contents could be lowered significantly to <40 %. Another option would be digesting BSG to produce biogas (Muster-Slawitsch et al., 2010). Thus, environmental and economic routes are needed for using BSG alternatively as raw material in different processes.
So far there has been little research on the conversion of BSG to bioethanol. Research on hydrolysis of spent grains for ethanolic fermentations has mainly focused on spent maize (corn), since this is a major byproduct of bioethanol plants in USA, claiming to have the potential to increase ethanol yields by 10 % in a corn mill (Grohmann and Bothast, 1997).
The first step in the bioethanol production process is the raw material pretreatment, aiming to improve permeability of vegetable cells and thus, dissolve hemicelluloses and make the cellulose more accessible to enzymes (Alvira et al., 2010). Pretreatment can be physical, chemical or biological (Banerjee et al., 2010), or a combination of these, depending on the characteristics of the feedstock and of the products that we want to obtain from it (Alvira et al., 2010). Hence, for complete conversion of the hemicelluloses and cellulose to monosaccharides, physical and/or chemical pretreatments coupled with enzymatic hydrolysis are required, since the lignin barrier, the complex structure of the cellulose and hemicellulose molecules, and the high crystallinity of lignocellulose restrict the enzyme action, inhibiting the direct hydrolysis with enzymes (Chandra et al., 2007).
Furthermore, fermentation of many celluloses and hemicelluloses in agricultural biomass is problematic because it primarily consists of pentoses (xylose and arabinose) that cannot be fermented by the yeast traditionally used in the alcohol industry (Saccharomyces cerevisiae) to produce ethanol from starch or glucose (hexose). As the hydrolysate contains both hexose and pentose sugars, it represents a challenge to yeast fermentation. Alternative yeasts, such as Pichia stipitis and Kluyveromyces marxianus have been used to ferment xylose (White et al., 2008) and Banat et al. (1996) demonstrated that not only the type of yeast (Kluyveromyces marxianus) but also the fermenter configuration and fermentation conditions have influence on bioethanol productivity. Candida auringiensis, Candida succiphila and Ambrosiozyma monospora can ferment arabinose (Dien et al., 1996) and combination of different yeasts can be used to ferment xylose to different products (Yablochkova et al., 2003). Also genetic modification of Saccharomyces cerevisiae has been investigated trying to improve fermentation of both arabinose (Becker and Boles, 2003) and xylose (Ito et al., 2010).
Therefore, according to this work objective, a parametric study of the biomass pretreatment and of the operational conditions used for enzymatic hydrolysis is important to ensure high monosaccharide recovery and thus, higher ethanol yields.

2. Acid pretreatment and enzymatic hydrolysis

This study intends to perform a parametric study of the acid pretreatment and enzymatic hydrolysis of BSG into simple sugars fermentable to ethanol. An innovation of this study is the realization of the pretreatment simultaneously with the hydrolysis. For this purpose were tested five enzymes (Viscozyme L, Cellulase and Hemicellulase from Aspergilus niger, Glucanex 100g and Ultraflo L) and three acids (HCl, HNO_3, H_2SO_4) added to BSG in one or two steps, which are described below in more detail. The concentration of the acids normally used in the pretreatments ranges from about 0.7 - 3.0 % (v/v) (Banerjee et al., 2010), being the most usual concentration of 1.0 % (v/v) that was used in this work.
Firstly, it was evaluated the action of Viscozyme L (Sigma Aldrich) for the hydrolysis of cellulose and hemicelluloses, simultaneously with the addition of two acids’ mixtures for the BSG pretreatment: HCl with HNO_3 (in one test) and HCl with H_2SO_4 (in other test). Therefore, to 25.0 g of BSG the mixture of desired

acids was added in the following quantities: [100 mL HCl, 1.0 % (v/v) with 100 mL HNO_3, 1.0 % (v/v)] in one test, and [100 mL HCl 1.0 % (v/v) with 100 mL H_2SO_4 1.0 % (v/v)] in other test. For each test the pH was adjusted to 5.0 (with NaOH 1 N) and then it was added 2.30 mL of Viscozyme L. This mixture was placed in a thermostatic bath at 50 °C and 75 rpm for 30 min, after which the sugars were evaluated by high performance liquid chromatography (HPLC) analysis.

The results obtained in these tests are summarized in Table 1 (trials 1 and 2), showing that the acids mixture that promoted the greater release of glucose plus maltose (dimer of glucose) from the BSG is HCl with H_2SO_4, being higher the amounts of xylose and arabinose for the mixture of HCl and HNO_3. In terms of total conversion of sugars it is slightly higher for the mixture of acids HCl with HNO_3. However this difference (2.8 wt%) is so small that does not allow one to conclude that this is the better acids' mixture to perform the pretreatment of BSG simultaneously with its hydrolysis by Viscozyme L.

Secondly, it was evaluated the action of the mixture of enzymes Cellulase and Hemicellulase from Aspergilus niger (Sigma Aldrich). These enzymes can be used in mixture since their optimum pH and temperature conditions are very close, according to the information from these enzymes' manufacturer, being respectively, pH 5.0 and 4.5 and 37 and 40 °C of temperature. Furthermore, according to other authors (Canilha et al., 2010) these enzymes can be used simultaneously thereby complementing their action of hydrolysis of cellulose and hemicelluloses. Thus, to 25.0 g of BSG the desired mixture of acids was added in the following quantities: [100 mL HCl, 1.00 % (v/v) with 100 mL HNO_3, 1.00 % (v/v)] in one test, and [100 mL HCl 1 % (v/v) with 100 mL H_2SO_4 1 % (v/v)] in other test. For each test the pH was adjusted to 4.8 (with NaOH 1 N) and then it was added the enzyme mixture containing 1.0 g of Cellulase and 1.0 g of Hemicellulase. This mixture was placed in a thermostatic bath at 39 °C and 75 rpm for 30 min, after which the sugars were analyzed by HPLC. The results obtained in these tests are summarized in Table 1 (trials 3 and 4), showing that the acids that promoted the greater release of glucose plus maltose (dimer of glucose) from the BSG is HCl with H_2SO_4, being greater the amounts of xylose and arabinose for the mixture of HCl and HNO_3. However the difference (3 wt%) is relatively small not allowing one to state that one acids' mixture is superior to the other for the pretreatment of BSG simultaneously with the enzymatic hydrolysis by Cellulase and Hemicellulase.

Briefly, in trials 1 to 4 the range of total sugars conversion (20-27 wt%) is so narrow to conclude about which is the best mixture of acids and enzymes, but in terms of the simple sugars obtained from BSG, the mixture of HCl and H_2SO_4 promoted the biggest release of maltose plus glucose whereas the mixture of HCl and HNO_3 promoted the greater release of xylose and arabinose. The presence of maltose in these trials is due to the incomplete hydrolysis of this sugar, once maltose is a dimer of glucose.

Thirdly, it was evaluated the action of the enzymes Glucanex 100g and Ultraflo L (Novozymes) and the result of using different amounts of each one. The choice of these enzymes was based on the optimization performed in a previous study (Caetano et al., 2011). Moreover, one also intended to determine whether the sequential addition of each acid (in two steps), instead of in mixture, would accrue significant advantages in terms of conversion. Thus, one started by evaluating the use of the two acids HCl and H_2SO_4 in sequence for performing the pretreatment simultaneously with the hydrolysis. For this purpose, to 25 g of BSG it was added 100 mL of HCl (with pH adjustment to 4.5) and the desired amount of Glucanex 100g. This mixture was placed in a thermostatic bath at 75 °C and 75 rpm for 30 min. After this time, to the resulting mixture it was added 100 mL of H_2SO_4 (with pH adjustment to 6.0) and the desired amount of Ultraflo L. This mixture was again placed in a thermostatic bath at 50 °C and 75 rpm for further 30 min. Finally, the mixture was allowed to cool and the simple sugars were analyzed by HPLC. The results obtained in these tests are summarized in Table 1 (trials 5 to 7).

Results from trials 5 to 7 show that the greatest conversion of total sugars (54.5 wt%) is obtained using higher amounts of Ultraflo L (2.30 mL) to similar amounts of Glucanex 100g (trial 6 in comparison to trial 5). Furthermore, it is also observed that when increasing the amount of Glucanex 100g to the same amount of Ultraflo L (trial 7 in comparison to trial 6) the total sugars conversion doesn't increase, rather, it decreases from 54.5 wt% to 40.5 wt%. On the other hand, increasing the amount of Ultraflo L (from trial 5 to trial 6) enhances the glucose release, while increasing the amount of Glucanex 100g (from trial 6 to trial 7) enhances the arabinose and maltose release.

Fourthly, it was evaluated whether the acids HCl and HNO_3 would improve the sugars conversion, by the action of Glucanex 100g and Ultraflo L, and also, which method would benefit the sugars conversion, if the acids sequential addition (in two steps) or in mixture (in one step). Therefore, in one test (trial 8 of Table 1), to 25.0 g of BSG was added the mixture of acids in the following quantities: [100 mL HCl, 1.0 % (v/v) with 100 mL HNO_3, 1.0 % (v/v)] (with pH adjustment to 4.5) and about 2.5 g of Glucanex 100g and placed in a thermostatic bath at 75 °C and 75 rpm for 30 min. Then, after pH adjustment to 6.0 it was added 2.30 mL of Ultraflo L and placed in a thermostatic bath at 50 °C and 75 rpm for further 30 min. Finally, the mixture was allowed to cool and the simple sugars were analyzed by HPLC.

In the other test (trial 9 of Table 1), to 25.0 g of BSG was added 100.0 mL of HCl 1.0 % (v/v) (with pH adjustment to 4.5) and about 2.5 g of Glucanex 100g. The mixture was placed in a thermostatic bath at 75 ºC and 75 rpm for 30 min. After this time, to the resulting mixture was added 100.0 mL of HNO_3 1.0 % (v/v) (with pH adjustment to 6.0) and 2.30 ml of Ultraflo L and placed in a thermostatic bath at 50 ºC and 75 rpm for more 30 min. Finally, the mixture was allowed to cool and the simple sugars were analyzed by HPLC.
As a result of the first test, it was observed a decrease (trial 8 in comparison to trial 7) in the total sugars conversion, particularly of maltose and glucose, the latter was not even detected. As a result of the second test (trial 9), the highest total sugars conversion was obtained (72.1 wt%), corresponding to 18 g sugars/ 25 g of dry BSG, being this value the maximum conversion obtained so far. Thus, one can conclude that the acids sequential addition (in two steps) is the best method to obtain higher conversions of sugars. One possible explanation is due to the dilution effect when using the acids in mixture that is not so significant when using the acids in a sequential addition, although initially the volume ratio of acid per amount of BSG is smaller, the acid concentration is higher.

Table 1: Summary of the results obtained in the several trials for evaluating the acid pretreatment simultaneously with the enzymatic hydrolysis of BSG

Trials	Acids	Enzymes	Glucose (g)	Xylose (g)	Arabinose (g)	Maltose (g)	Total sugars (wt%)
1	Mix ($HCl+H_2SO_4$) pH 5.0	2.30 mL Viscozyme L	0.848	1.537	2.771	1.893	20.6
2	Mix ($HCl+HNO_3$) pH 5.0	2.30 mL Viscozyme L	0. 933	1.757	2.816	0.339	23.4
3	Mix ($HCl+H_2SO_4$) pH 4.8	Mix (1.0 g Cellulase+1.0 g Hemicellulase)	1.189	1.406	2.380	1.708	26.7
4	Mix ($HCl+HNO_3$) pH 4.8	Mix (1.0 g Cellulase+1.0 g Hemicellulase)	1.385	1.577	2.742	0.216	23.7
5	Seq. (HCl pH 4.5 / H_2SO_4 pH 6.0)	Seq (1.51 g Glucanex 100g+1.75 mL Ultraflo L)	0.726	0.252	1.377	0.313	10.7
6	Seq. (HCl pH 4.5 / H_2SO_4 pH 6.0)	Seq (1.67 g Glucanex 100g+2.30 mL Ultraflo L)	8.758	1.625	2.920	0.315	54.5
7	Seq. (HCl pH 4.5 / H_2SO_4 pH 6.0)	Seq (2.48 g Glucanex 100g+2.30 mL Ultraflo L)	1.608	1.708	4.338	2.473	40.5
8	Mix ($HCl+HNO_3$) pH 4.5	Seq. (2.54 g Glucanex 100g/ 2.30 mL Ultraflo L)	1.350	1.813	4.246	-	29.6
9	Seq. (HCl pH 4.5 / HNO_3 pH 6.0)	Seq (2.48 g Glucanex 100g/ 2.30 mL Ultraflo L)	4.108	3.638	10.280	-	72.1

3. Fermentation of sugars to bioethanol

After the BSG pretreatment and hydrolysis, the next step for bioethanol production is the sugars fermentation to ethanol. Thus, in this work one intended to evaluate the efficiency of sugars conversion to ethanol. The yeast used for that purpose is Saccharomyces cerevisiae, known as baker's yeast (with the commercial brand Levital). Although it is known from literature that this yeast alone is not able to ferment pentoses (Erdelji, 2007), it was used in this work to get an idea of the results that would be achieved, particularly regarding glucose fermentation. Therefore, a certain amount of yeast, determined by theoretical relationship (Soares, 2006), was added to each assay. The pH of 5.5 was adjusted (with NaOH 1.0 N) and the mixture placed in a thermostatic bath at 30 °C and 75 rpm for 48 h, during which samples were taken to analyze the evolution of sugars fermentation by HPLC.
Figure 1 presents the results of fermentation of the sugars obtained in trials 7 and 9 of Table 1, for which the fermentation efficiency was respectively 89.6 and 78.5 %.
As shown in Figure 1, the amount of each sugar not always reduced, which may be due to an experimental error in determining the concentration or conversion of each sugar. Other explanation is that during the fermentation process there may have been conditions that enabled the action of enzymes and a further hydrolysis process (as if there was a fermentation and simultaneous saccharification).
The initial amount of sugars markedly decreased; in particular with respect to glucose and maltose (maltose is a dimer of glucose). Maltose was fully fermented, but for glucose the full conversion didn't occur, perhaps requiring a longer fermentation time (over 48 h). Although Saccharomices cerevisiae is

known to ferment hexoses rather than pentoses, the initial amounts of xylose and arabinose decreased to a certain extent. The introduction of another enzyme would be beneficial for a complete fermentation of all the sugars.

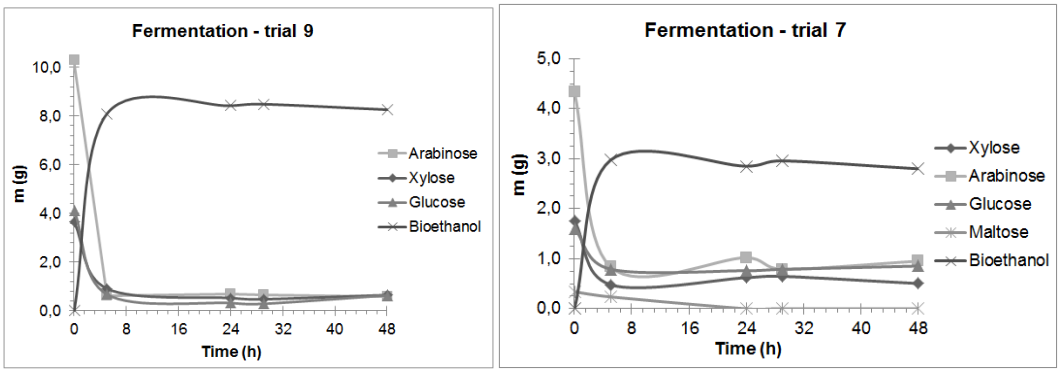

Figure 1: Evolution of the amount of sugars and ethanol in the fermentation mash along 48 h

By knowing the amount of the four sugars (glucose, xylose, arabinose, and maltose) consumed in each test, and following the stoichiometry of their fermentation reactions, it was possible to calculate the theoretical mass and volume of ethanol to be obtained from the sugars of trials 7 and 9 of Table 1. This is 8.3 g and 5.10 mL for the fermentation of the trial 9's sugars and 4.1 g and 5.2 mL for the fermentation of the trial 7's sugars. These values were not validated experimentally because the quantities of BSG used in each assay were so small (25 g) that the losses associated with the experimental process of ethanol recovery by distillation would be affected by a great experimental error.
Although in this study the fermentation was not complete, it was obtained about 419 mL ethanol/ kg dry BSG from 720 g sugars / kg dry BSG (of which 144 g xylose, 410 g arabinose and 164 g glucose) for the best total sugars conversion (72.1 wt%). Compared to other common feedstock normally used to produce bioethanol, one can obtain 720 g sugars from 1 kg of dry BSG, being this value higher than the one reported in the literature for corn (550 g sugars / kg corn), identical to the one for corn straw (720 g sugars/ kg straw), lower than the value for sugarcane (830 g sugars / kg sugarcane) that is currently the most developed industrially (Caetano et al., 2012), and much higher when compared to the one for lignocellulosic materials such as wood sawdust (282 - 316 g total sugars / kg WSD, Caetano et al., 2009).

4. Conclusions

This work performed a parametric study aiming the optimization of the acid pretreatment of BSG together with the enzymatic hydrolysis, having in mind the production of bioethanol. Considering that each acid has different potential on sugars release, the pretreatment was accomplished by adding two acids either in mixture (in one step) or sequentially (in two steps) to 25 g dry BSG, followed by the action of different enzymes. The pretreatment efficiency was evaluated by quantifying the sugars release after hydrolysis.
Results have shown that the sequential addition of acids is the best method to obtain greater conversions of sugars, regardless the acids used. In particular, the pretreatment of BSG with HCl and HNO_3 added sequentially, together with the hydrolysis by Glucanex 100 g and Ultraflo L, resulted in the best yield in terms of BSG conversion to simple sugars, corresponding to about 720 g of sugars per kg of dry BSG. This value is very interesting when compared to the yield of sugars obtained from common bioethanol feedstocks, allowing one to conclude that BSG is a material with a great interest for bioethanol production.

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