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Bioethanol from Brewers' Spent Grain: Pentose Fermentation

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This work aims to perform a preliminary optimization of the fermentation of brewers' spent grain (BSG) sugars to bioethanol, focusing on the pentose sugars. Firstly, it was conducted the acid pre-treatment and enzymatic hydrolysis for studying different reaction times and amounts of enzymes in order to convert cellulose and hemicelluloses into simple sugars. The greatest amount of total sugars achieved experimentally is 5.56 g/ 25 g of dry BSG, corresponding to a sugars maximum conversion of 22.24 % from a BSG sample with about 6 % cellulose and about 40 % hemicelluloses. This was obtained by sequentially adding the acids HCl and HNO3 with 1 wt% of concentration to BSG to perform the pre-treatment, simultaneously with 1.0657 g of Glucanex 100g and 2.0 mL of Ultraflo L for the hydrolysis. Secondly, it was studied the fermentation step, using both synthetic medium and BSG hydrolyzate, and the yeasts *Pichia stipitis* NCYC 1541 (*P. stipitis*) and *Kluyveromyces marxianus* NYCY 2791 (*K. marxianus*). Results show that the fermentation efficiency of all sugars in the synthetic media is higher than 80_% for both yeasts, but in the BSG hydrolyzate it is just 45.10 % for *P. stipitis* and 36.58 % for *K. marxianus* for a 72 h fermentation time at a 30 °C temperature. The theoretical ethanol yield from BSG hydrolyzates is 0.27 and 0.19 g ethanol/ g of sugars for respectively, *P. stipitis* and *K. marxianus*, but the actual ethanol yield obtained in this work is 0.0856 and 0.0308 g ethanol/ g of sugars, respectively that is three times smaller than the theoretical yield for *P. stipitis* and six times smaller for *K. marxianus*, which can be attributed to the presence of inhibitors resulting from the previous steps.

1. Introduction

The utilization of lignocellulosic biomass is one of the most promising alternatives for liquid biofuels to be used directly in the vehicle engine (Caetano et al., 2014) or as raw material for the production of other biofuels (Mata et al., 2012a), with environmental, societal and economic benefits (Mata et al., 2013). Bioethanol production from lignocellulosic biomass is complex due to its structural properties and requires the utilization of the carbohydrate fractions, i.e. cellulose and hemicelluloses. The production process involves several steps including the biomass pre-treatment, hydrolysis of carbohydrates, fermentation of simple sugars into ethanol, and distillation for product recovery (Caetano et al., 2013).
BSG is a lignocellulosic material basically consisting of the husk of barley grain. It is the major by-product generated by the brewing industry, representing around 85 % of the total by-products (Mussatto et al., 2006). The BSG chemical composition varies depending on the barley variety and harvest time, and on the brewing process conditions. 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). So far, the main destination for BSG has been cattle feeding, due to its high content of protein and fiber (Caetano et al., 2013). However this application has limited market value and other uses are under research, such as bioethanol production, which can be used as fuel (Mata et al., 2003) or blended in gasoline (Mata et al., 2005) and as raw material for other biofuels production (Caetano et al., 2012). Bioethanol production from BSG is considered a 2nd generation biofuel process since it has no direct conflict with human food, as it happens with

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the 1st generation biofuels produced from agricultural crops, such as corn and sugarcane for bioethanol production, or soybean oil (Morais et al., 2010) or corn oil (Mata et al., 2012b) for biodiesel production.
In previous studies conducted by the authors of this work it was determined the best operating conditions to perform the acid pre-treatment and the enzymatic hydrolysis of BSG (Caetano et al., 2013), which consist of doing an acid pre-treatment with the sequential addition of HCl and HNO_3, with 1 wt% of concentration, and adding the enzymes Glucanex 100g and Ultraflo L simultaneously with the acids to perform the hydrolysis. This method 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. As the BSG hydrolyzates contains both hexose and pentose sugars, it represents a challenge to yeast fermentation. Thus, in this work further experiments were conducted aiming to perform a preliminary optimization of the fermentation step, focusing on the pentose sugars (ribose, xylose and arabinose), which cannot be fermented by the yeast traditionally used in the alcohol industry (*Saccharomyces cerevisiae*) to produce ethanol from starch or glucose (hexose sugars). In fact few microorganisms are known, which are capable of pentose metabolism (Kuhad et al., 2011). Therefore, in this study the alternative yeasts *Pichia stipitis* NCYC 1541 and *Kluyveromyces marxianus* NYCY 2791 were used aiming the pentose fermentation, as proposed by White et al. (2008) and by Banat et al. (1996).

2. Materials and methods

To accomplish this work objectives a set of experimental studies were conducted, including the physical and chemical characterization of BSG, acid pre-treatment, enzymatic hydrolysis, pentose fermentation, and ethanol determination.

2.1 Characterization of brewers' spent grain

For this study, the BSG were collected from a local brewing company. The composition of BSG varies with several factors, in particular, harvest time and type of beer to be produced. Thus, the parameters evaluated include: moisture and ash content, particle size, higher heating value, lipid, cellulose, hemicelluloses, and lignin contents, and total organic carbon.
The moisture content is determined by repeated cycles of oven (WTB Binder) drying at 105 ± 5 ºC for 2 h followed by cooling in a desiccator and weighing until a constant weight following the procedure of standard method 2540D (APHA,1999).
The ash content is determined using a furnace (Nabertherm B150) at 550 ±5 ºC for 4 h, following the procedure of standard method 2540E (APHA, 1999).
The particle size classification is done using a sieve system with meshes of different openings' sizes and respective vibrator. The masses of the fraction collected in each sieve are recorded.
The higher heating value is determined using an oxygen bomb calorimeter (Parr, 6772) following the standard method CSN EN 14918 (2009).
The lipid content is determined by solid-liquid extraction using the Soxhlet method, following the procedure described in the standard NP 1005 (1974).
The cellulose content is determined using a heating plate (SELECTA, Agimatic-E), a vacuum filtration apparatus and a vacuum pump (Neuberger), an analytical balance (Kern ALJ 220-4), a furnace (Vulcan A-550) and an oven (WTB Binder), following the procedures described in the standard NP 1005 (1974).
The hemicelluloses content is determined using a Soxhlet extraction system, by adding 200 mL NaOH solution 2 % (w/v) to a 1 g BSG sample and allowing it to boil for 4 h. After this time the material is filtered and washed with deionized water, and placed in an oven at 105 °C to constant mass. The hemicellulose content is determined by the ratio of the final mass to the mass of BSG initially weighed.
The insoluble (or Klason) and soluble lignin content is determined following the procedure of the standard TAPPI T222om-02, using a heating plate (SELECTA, Agimatic-E) and a UV-Vis spectrophotometer (Shimadzu, UV-1700 pharmaspec) to measure the absorbance of the filtered solution resulting from the previous procedure at a 205 nm wavelength.
The total organic carbon is determined using a SHIMADZU, TOC-VCSN Analyzer.

2.2 Pre-treatment and enzymatic hydrolysis

The acid pre-treatment and enzymatic hydrolysis are carried out in thermostatic bath, at 50 °C, with stirring at 75 rpm. Different reaction times and different ratios of enzyme/BSG were tested, using the same amount (100 mL) of acids (HCl and HNO_3) at 1 % (w/w) concentration, according to the flow diagram of *Figure 1*. In all tests the pH is adjusted to 4.6 for the enzyme Glucanex 100g and to 6.0 for enzyme Ultraflo L.

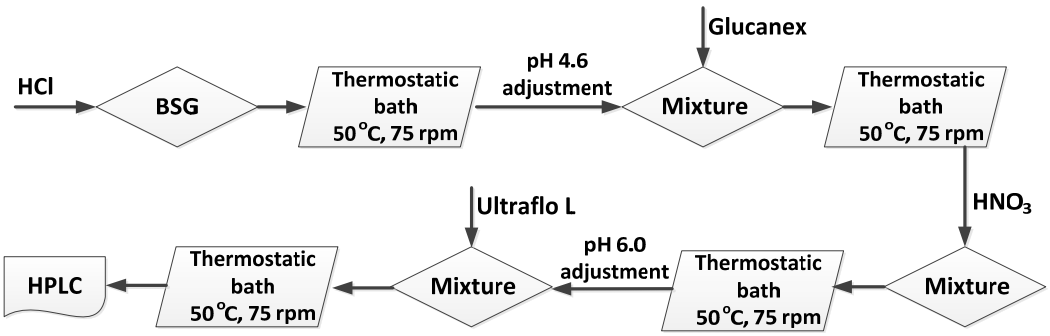

Figure 1: Process diagram of the pre-treatment and enzymatic hydrolysis

2.3 Preparation of yeasts cultures for fermentation

For conversion of the sugars resulting from the acid pre-treatment and enzymatic hydrolysis into bioethanol, two yeast strains were used: *Pichia stipitis* NCYC 1541 and *Kluyveromyces marxianus* NCYC 2791, acquired from the National Collection of Yeast Cultures (NCYC), UK. The inoculum of yeast biomass to be used in the fermentation trials is prepared using the culture medium Yeast Extract Peptone Dextrose (YEPD). Both liquid and solid YEPD media were prepared for the cultures' growth and conservation, respectively.
The YEPD liquid culture medium is prepared by dissolving 10 g of yeast extract, 20 g peptone, and 20 g glucose in 1000 mL of water. The YEPD solid culture medium is prepared using 3.9 g of yeast extract, 2.0 g peptone, 3.9 g glucose and 4.1 g agar in 200 mL of water. The yeast cultures are grown in YEPD liquid and incubated at 25 °C for 3 days and the subculture is also grown in an incubator at 25 °C for 2 days. The yeasts are maintained in test tubes in the YEPD solid at 3 °C of temperature. All the material used in the preparation of the culture media, yeast cultures and subcultures, are sterilized by autoclaving at 121 °C for 20 min. Before fermentation, the BSG hydrolyzates were vacuum filtered using glass fiber membranes for removing the solids, and then sterilized by autoclaving at 121 °C for 20 min.

2.4 Fermentation conditions

The fermentation process is done in thermostatic bath, at 30 °C, with stirring at 75 rpm, for a 72 h period.
All assays are performed with 10% (v/v) of inoculum, using both synthetic medium (*Table 1*) and BSG hydrolyzates in order to compare the fermentation results and to evaluate the potential effects of inhibitors resulting from the pre-treatment and hydrolysis of BSG. Two replicates were performed for each yeast and fermentation medium.

Table 1: Composition of synthetic medium

Sugars	Ribose	Xylose	Arabinose	Maltose	Glucose
Mass (g)	12.8855	15.806	9.6881	8.2929	12.4563

Samples were taken periodically for measurement of the sugars consumption by HPLC. The HPLC is composed by a 231XL injection valve (Gilson) with a sampling loop of 20 µL and a Gilson pump (model 307), equipped with a detector of type Evaporative Light Scattering (ELSD) (PLEMD 960 model, Polymer Laboratories), under the following conditions: air flow of 7 mL/min, detector temperature of 70 °C, and 50 bar of pressure in the column. The eluent used is a mixture acetonitrile/ ultrapure water, in proportions of 80/20 (v/v), at a 0.7 mL/min flow rate. The column used is a Knauer Eurospher II 100, NH2, 5 µm, 250x4.6 mm, with pre-column at room temperature. The analysis time is 40 min.

2.5 Recovery and quantification of bioethanol

Due to the low volumes of alcohol obtained experimentally it was not possible to carry out a distillation for its recovery. Thus, for the ethanol content quantification it was employed the analytical method Antan Paar. This system consists of a density meter DMA 4500, with an oscillating U-tube, a metering module *Alcolyzer Beer* ME, and in the sample tracer an *Xsample* 122. The NIR measurement method (reflecting near infrared region) eliminates the influence of other sample components in the alcohol content measurement and guarantee high accuracy results.

3. Results and discussion

3.1 Characterization of brewers' spent grain

Table 2 presents the results from the BSG characterization.

Table 2: Characterization of brewers' spent grain

Parameter	Value
Particle size (mm)	0.149-1.190
Moisture (%)	72.0
Ash (%)	4.4
Higher heating value (MJ/kg)	19.8
Lipid content (%)	5.4
Cellulose (%)	6.09
Hemicelluloses (%)	39.7
Lignin content (%)	34.8
Total organic carbon (%)	97.9

All % values are on dry weight basis, except moisture content.

3.2 Study of pre-treatment and enzymatic hydrolysis

To evaluate the pre-treatment and enzymatic hydrolysis efficiency, the sugars released from 25 g of dry BSG were quantified by HPLC in each experiment, studying different acids and enzymes' contact times and enzymes' amounts. *Table 3* and *Table 4* show the results of experiments 1 to 6.

Table 3: Results of pre-treatment and enzymatic hydrolysis in experiments 1 to 3

Sugars	Experiment 1		Experiment 2		Experiment 3	
	Composition % (wt/wt)	Mass (g/25 g BSG)	Composition % (wt/wt)	Mass (g/25 g BSG)	Composition % (wt/wt)	Mass (g/25 g BSG)
Ribose	59.49	2.29	56.51	1.61	53.79	1.61
Xylose	14.35	0.55	0.00	0.00	9.52	0.28
Arabinose	0.00	0.00	22.02	0.63	21.55	0.64
Maltose	19.80	0.76	13.79	0.39	6.95	0.21
Glucose	6.36	0.24	7.68	0.22	8.18	0.24
Total	100	3.84	100	2.85	100	2.98

Exp 1: HCl, HNO_3, Glucanex 100 g and Ultraflo L contact times 30 min for each one; $m_{Glucanex}$= 0.5 g; $V_{Ultraflo\ L}$ = 0.5 mL
Exp 2: HCl, HNO_3, Glucanex and Ultraflo L contact times 30, 60, 30, 30 min, $m_{Glucanex}$= 0.5 g; $V_{Ultraflo\ L}$ = 0.5 mL
Exp 3: HCl, HNO_3, Glucanex and Ultraflo L contact times 60, 60, 30, 30 min, $m_{Glucanex}$= 0.5 g; $V_{Ultraflo\ L}$ = 0.5 mL

Table 4: Results of pre-treatment and enzymatic hydrolysis in experiments 4 to 6

Sugars	Experiment 4		Experiment 5		Experiment 6	
	Composition % (wt/wt)	Mass (g/25 g BSG)	Composition % (wt/wt)	Mass (g/25 g BSG)	Composition % (wt/wt)	Mass (g/25 g BSG)
Ribose	65.91	3.67	57.72	1.84	55.09	2.16
Xylose	5.95	0.33	0.00	0.00	8.52	0.33
Arabinose	16.75	0.93	24.09	0.77	23.10	0.90
Maltose	6.99	0.39	9.67	0.31	5.50	0.22
Glucose	4.39	0.24	8.52	0.27	7.78	0.30
Total	100	5.56	100	3.19	100	3.91

Exp 4: contact times of HCl, HNO_3, Glucanex and Ultraflo 60, 60, 30, 30 min, $m_{Glucanex}$= 1.0657 g; $V_{Ultraflo}$ = 2.0 mL
Ex 5: contact times of HCl, HNO_3, Glucanex and Ultraflo 30, 30, 30, 60 min, $m_{Glucanex}$= 1. 5464 g; $V_{Ultraflo}$ = 2.3 mL
Exp 6: contact times of HCl, HNO_3, Glucanex and Ultraflo 420, 960, 420, 420 min, $m_{Glucanex}$= 0.5228 g; $V_{Ultraflo}$ = 0.5 mL

Tables 3 and 4 show that the increase in contact times does not favor the sugars conversion. In particular a 30 min contact time for the enzymes is enough to complete the hydrolysis reaction. Similarly, the increase of the enzymes amount favors the sugars conversion just to a certain point. Ribose and arabinose are the simple sugars with the highest production rate. The highest conversions of total pentose sugars (ribose, xylose and arabinose) were obtained in experiments 4 (19.72 %) and 6 (13.56 %). The highest conversions of total hexose sugars (maltose and glucose) were obtained in experiments 1 (4.00 %) and 4 (2.52 %). The low conversion of hexoses is due to the low cellulose content (6 %) of the BSG sample used in this work.The highest total sugars conversion occurred in experiment 4, in which ribose and arabinose are approximately 66 % and 17 % of the total sugars, corresponding to respectively, 3.7 g and 0.9 per 25 g of dry BSG. However, experiment 1 has the highest conversion rate of xylose and maltose sugars together, being 14.35 % and 19.80 % of the total sugars or 0.55 and 0.76 g per 25 g of dry BSG, respectively.
The greatest total sugars conversion of 22.24 % was obtained in experiment 4, corresponding to 5.56 g sugars per 25 g BSG, followed by experiment 6 (15.64 % or 3.91 g sugars/ 25 g BSG) and experiment 1 (15.36 % or 3.84 g sugars/ 25 g BSG). In particular in experiment 4, ribose and arabinose are 65.91 % and 16.75 % of the total sugars, corresponding to 3.67 and 0.93 g per 25 g of dry BSG, respectively.
The maximum sugars conversion reported in previous studies is 72 % (Caetano et al., 2013), from a BSG sample with about 13 % cellulose and using 2.5 g of Glucanex 100g and 2.3 mL of Ultraflo L. In the present study, the sugars maximum conversion is 22.24 % (in experiment 4) obtained from a 25 g BSG sample with 6 % cellulose and using 1.1 g of Glucanex 100g and 2.0 mL of Ultraflo L. The sugars maximum conversion is lower in this study in comparison with the previous studies because the BSG sample used contains less than half the amount of cellulose. Although the maximum sugars conversion of 22.24 % was obtained in experiment 4, the highest conversion efficiency of the polysaccharides into simple sugars was obtained in experiment 1, in which a lower amount of enzymes and a shorter reaction time were employed.

3.3 Fermentation study

After selecting the best operating conditions for the pre-treatment and enzymatic hydrolysis, the fermentation step was studied using both synthetic medium and BSG hydrolyzates (*Table 5*).

Table 5: Sugars conversion by fermentation in synthetic medium and BSG hydrolyzates

Sugars conversion	Synthetic medium		BSG hydrolyzates	
% (wt/wt)	*P. stipitis*	*K. marxianus*	*P. stipitis*	*K. marxianus*
Xylose	91.47±0.07	89.59±2.62	58.00±12.35	46.46±5.98
Arabinose	59.30±10.33	61.82±14.05	22.80±6.02	15.35±3.04
Ribose	82.01±7.04	80.84±1.50	-	-
Maltose	83.97±0.02	83.93±0.07	55.00±9.80	41.46±4.32
Glucose	93.97±2.10	92.52±0.01	26.00±12.41	27.42±13.77
Average	83.61±2.80	83.74±0.15	45.10±9.70	36.58±2.49

Results show that the highest sugars consumption occurred in the first 40 h, from which the sugars concentrations remained fairly constant. In the synthetic medium, the overall fermentation efficiency of synthetic sugars after 72 h, is around 83 % for both yeasts, with a lower efficiency for arabinose, corresponding to an estimated ethanol production of 25 g from 59 g of total sugars. White et al. (2008) found that *P. stipitis* is capable of converting more than 60 % of synthetic sugars by fermentation in 48 h, while *K. marxianus* only converts 30 %. The fermentation efficiency of the synthetic liquor is in average 91 % to xylose and 60 % to arabinose by both yeasts. In the BSG hydrolyzates, both yeasts are able to consume all sugars, but with a better performance of *P. stipitis* that consumed around 45,10 %, while *K. marxianus* around 36.58 %, corresponding to an estimated ethanol production of respectively, 3.2 and 2.2 g ethanol per 12.23 g of sugars obtained from 100 g of dry BSG. The fermentation efficiency of the BSG hydrolyzates is in average 58.00 % to xylose and 22.80 % to arabinose by *P. stipitis*, and 46.46 % to xylose and 15.35 % to arabinose by *K. marxianus*. In the BSG hydrolyzates, the theoretical ethanol yield is 0.27 and 0.19 g ethanol /g of sugars for *P. stipitis* and *K. marxianus* respectively, and the actual ethanol yield is 0.0856 and 0.0308 g ethanol/ g of sugars for *P. stipitis* and *K. marxianus* respectively, which is three times smaller than the theoretical yield for *P. stipitis* and six times smaller for *K. marxianus*. White et al (2008) determined higher values for the actual ethanol yield of 0.32 g and 0.22 for respectively, *P. stipitis* and *K. marxianus*. This can be explained by the type of pentose sugars present in this work and also, by the presence of fermentation inhibitors generated during the pre-treatment and hydrolysis steps. The ethanol content determined experimentally using the Antan Paar system in the 600 mL fermentation wort is 0.22 and

0.08 % (v/v) for respectively *P. stipitis* and *K. marxianus*, corresponding to ethanol volume of 1.32 and 0.48 mL, or an ethanol mass of 1.054 g e 0.378 g, for respectively *P. stipitis* and *K. marxianus*.

4. Conclusions

In this work preliminary optimization tests of BSG hydrolyzates fermentation were conducted by the yeasts *P stipitis* and *K. marxianus*, focusing on pentose sugars. The acid pre-treatment and enzymatic hydrolysis steps were firstly applied, for studying the reaction times and amounts of enzymes, in order to determine the best operating conditions for the highest conversion efficiency of polysaccharides into simple sugars. The greatest amount of total sugars obtained experimentally is 5.56 g/ 25 g BSG, corresponding to a sugars maximum conversion of 22.24 %, by doing the acid pre-treatment and enzymatic hydrolysis with 1.0657 g Glucanex and 2.0 mL Ultraflo. Secondly, the fermentation step was studied using both synthetic medium and BSG hydrolyzates for comparing the fermentation results and evaluating potential effects of inhibitors from the previous process steps. Overall, *P. stipitis* performs better than *K. marxianus* for converting the BSG hydrolyzate sugars. The sugars conversion efficiency in the synthetic medium is above 83 % for both yeasts and in the BSG hydrolyzates it is around 45 % for *P. stipitis* and around 37 % for *K. marxianus.* In the BSG hydrolyzates the theoretical ethanol yield is 0.27 and 0.19 g ethanol /g of sugars for *P. stipitis* and *K. marxianus* respectively, which is higher than the actual ethanol yield of 0.0856 and 0.0308 g ethanol/ g of sugars respectively, probably due to the present of fermentation inhibitors (e.g. acetic acid, furfural, hydroxymethylfurfural) resulting from the previous process steps.

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