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# Brewer´s Spent Grain to Bioethanol through a Hybrid Saccharification and Fermentation Process

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Brewer´s spent grain, without being pre-treated, has been investigated for bioethanol production through a Hybrid Saccharification and Fermentation (HSF) process with high solid loading. HSF experiments were performed in a 2 L bioreactor where Cellic ® CTec2 was used to perform the enzymatic hydrolysis, and *Saccharomyces Cerevisiae* was used for the fermentation. The reaction environment was first set to favour saccharification. Then, after 26 h, the reactor was inoculated with the yeast. The results evidenced the presence of glucose, xylose, and arabinose after the conversion of cellulose and hemicellulose and a rapid depletion of glucose after adding the yeast. The pentoses were also consumed, but with a much slower reaction rate. Almost four hours after adding the yeast, the amount of ethanol had reached a maximum and then began to decrease as microorganisms began to use ethanol as a substrate after glucose depletion. The obtained ethanol yield, evaluated with respect to the theoretical value, was equal to 72%.

## 1. Introduction

Brewer's spent grain (BSG) is the major by-product generated by breweries. It is estimated that 20 kg of BSG outcomes the lautering unit per 100 L of beer produced (Mussatto et al., 2006). There are two primary uses for this biowaste: animal feed and land disposal (Lisci et al., 2022). Nevertheless, its cellulose content makes it a valuable feedstock to produce second-generation ethanol, offering a good alternative to petroleum-derived chemicals and reducing the food versus fuel conflict generated by first-generation ethanol. BSG mainly consists of three polymeric fractions: cellulose, hemicellulose, and lignin (Mussatto et al., 2008). Such compounds form a complex and recalcitrant fibrous structure, so a process of separation and lysis of BSG's polysaccharides is required to give ethanologenic microorganisms access to fermentable sugars. For this purpose, different studies reported the optimization of alkali and/or acid pretreatments required to degrade the lignin and the hemicellulose fractions (Mussatto, 2014). In such a way, the subsequent enzymatic hydrolysis process is enhanced since the access of enzymes to cellulose chains in a deconstructed lignocellulosic matrix is more feasible, giving rise to higher glucose yields (Pinheiro et al., 2019). On the other hand, the pretreatment of raw BSG implies several disadvantages related to the high cost of acid recovery and the cost related to corrosive fluid-resistant equipment (Solarte-Toro et al., 2019). Moreover, C5 (i.e. xylose and arabinose) and C6 (mainly glucose) in an acid environment are decomposed into furfural and hydroxymethylfurfural, which may inhibit both the enzymatic and metabolic activity (Rojas-Chamorro et al., 2020). Due to this, a detoxification stage may be required, thus increasing capital and operating costs.
The enzymatic hydrolysis of non-pretreated BSG represents an attractive alternative to produce fermentable sugars. However, the main issue of this alternative is related to the high viscosity of the slurry when high substrate concentrations are involved in the process (Mussatto et al., 2008). Indeed, mass transfer limitations are present, leading to lower glucose production rates so that ethanol generation is negatively affected. Furthermore, the operative window for the enzymatic hydrolysis may not be overlapped with the corresponding one for the yeast growth. Such issues may be overcome by initiating the enzymatic hydrolysis step before yeast inoculation, after which simultaneous saccharification and fermentation (SSF) occur.

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In this process configuration, liquefaction of the whole slurry helps reduce the viscosity (Pinheiro et al., 2019) and allows operation at higher temperatures during the first enzymatic hydrolysis stage; subsequent SSF provides survival conditions for microorganisms. This procedure is called hybrid saccharification and fermentation (HSF) (Cassells et al., 2017).
In this work, the less investigated enzymatic saccharification of untreated BSG using Cellic® Ctec2 was considered in order to assess the enzymatic hydrolysis performances of strongly recalcitrant biomass. The reaction environment was then modified to be suitable for yeast growth by appropriately adjusting pH and temperature. Finally, the slurry rich in sugars was fermented using *Saccharomyces Cerevisiae* for ethanol production.

## 2. Materials and methods

The BSG used in the experiments was kindly provided by Vestfyen brewery (Assens, Denmark). The wet raw material was initially stored at -20 °C and then oven-dried at 90 °C in a humidity-controlled oven (Memmert HCP 108). Drying samples were weighed every 2 hours until a constant weight was reached. Each sample had an initial weight of 100 g, and the time required to achieve a constant weight was 10 hours. BSG solid content was 25% wt. Dried BSG was then sealed and stored at room temperature for further experiments. In the following text, the notation raw/untreated BSG is intended as the biomass obtained after the drying step and without being further pre-treated with any chemicals.

### 2.1 Chemical composition analysis of the raw BSG

The chemical composition of raw material was determined according to National Renewable Energy Laboratory (NREL) standard methods. In particular, ash content was determined by means of NREL/TP-510-42622 (Sluiter et al., 2005) standard method, while NREL/TP-510-42618 (Sluiter et al., 2008) was used to determine the glucan, xylan, arabinan and lignin content of raw BSG. It is worth highlighting that a fair amount of solubilised glucose was identified at the operative temperature before the enzyme introduction. This amount of soluble glucose polysaccharide (SGP) may derive from unconverted starch and free glucose monomers (Pinhero et al., 2019). SGP mass fraction was determined by HPLC quantification of a liquid sample withdrawn 30 minutes after the temperature reached the operative value and before introducing the enzyme into the system. The average chemical composition is summarised in Table 1.

*Table 1: Composition of dry BSG. Values are reported as average value ± standard deviation*

| Component | BSG composition (wt % dry mass) |
|---|---|
| Glucose | 26.8 ±2.2 |
| of which SGP | 2.24±0.01 |
| Xylose | 24.0 ±2.0 |
| Arabinose | 11.8 ±1.0 |
| Acid soluble lignin | 9.9 ±1.6 |
| Acid insoluble lignin | 5.5 ±1.6 |
| Ash | 3.6 ±0.1 |
| Others[a] | 18.4 |
| Total mass | 100 |

[a] Other components may include proteins and extractives. Value obtained by difference.

### 2.2 Enzymatic saccharification of untreated BSG

The experiment was conducted in an autoclavable glass bioreactor of 2 L capacity (Applikon® Bioteknology, Netherlands) under anaerobic conditions. For this purpose, nitrogen was fed at 1 L/min. Rushton disk turbine (Applikon® Bioteknology, Netherlands) agitator was used to stir the system (250 RPM). Cellic ® CTec2 (>1000 Biomass Hydrolysis Units, BHU-2 g -1, density of 1.209 g/L) (Novozymes, Denmark) was used to perform the enzymatic hydrolysis of raw BSG. This enzyme complex comprises a blend of $\alpha$-cellulase, β-glucosidases and hemicellulases. Enzymatic pre-hydrolysis was performed at 49 °C, with the enzyme dosage equal to 0.22 ml/g dry BSG and the liquid-solid ratio equal to 15 (g/g), corresponding to 6.3 % wt. Such conditions had been obtained by a set of experiments aimed to maximise glucose yield, not reported in this paper. The reaction was performed in a citrate buffer of 0.05 M (pH 4.8) (Mussatto et al., 2008). Samples were withdrawn at different times to monitor BSG saccharification. The saccharification process was carried out for 24 hours to allow an

adequate release of fermentable sugars. The performances of the enzymatic process were evaluated by means of the glucose yield $Y_G$ (g of glucose per 100 g of potentially releasable glucose) calculated as follows:

$$Y_G = \frac{C_G - \frac{V_{enz}\rho_{enz}x_{G,enz}}{V_R}}{x_G C_{BSG}} \cdot 100 \quad (1)$$

Where $C_G$ is the glucose concentration after 24 hours from the beginning of the experiment, $V_{enz}$ is the volumetric amount of enzyme introduced in the reactor, $\rho_{enz}$ is the enzyme density, $x_{G,enz}$ is the glucose mass fraction in the enzymatic blend, $V_R$ is the reactor volume and $C_{BSG}$ is the dry BSG concentration. Finally, $x_G$ that is the glucose mass fraction in dried BSG was calculated by means of BSG glucan content $x_{Gn}$, multiplied by the stoichiometric factor for its hydration upon hydrolysis according to Eq(2)

$$x_G = \frac{180}{162} x_{Gn} \quad (2)$$

It is worth specifying that glucose content in the commercial enzyme mixture was quantified by HPLC and subtracted from the final glucose concentrations in order to properly assess the amount of glucose generated by the saccharification process. This amount of glucose is taken into account in the second term of the numerator reported in Eq(1). Precisely, the glucose mass fraction in the enzymatic bend was equal to $0.261 \pm 0.006$ while no xylose and arabinose were detected. The experiment was performed in duplicate.

### 2.3 Yeast and inoculum preparation

A commercial strain of *Saccharomyces Cerevisiae* (Malteserkors, Denmark) was used to set up the inoculum. During inoculum preparation, 1.5 g of dry yeast was pitched in 500 ml narrow mouth bottles capped with rubber stopper, filled with 150 mL of growth medium and left to grow overnight at 30 °C under anaerobic conditions in an orbital shaker at 150 RPM. Growth medium, stored at 4 °C until use, had the following composition: 20 g $L^{-1}$ glucose; 6 g $L^{-1}$ yeast extract; 0.23 g $L^{-1}$ $CaCl_2 \cdot 2H_2O$; 4 g $L^{-1}$ $(NH_4)_2SO_4$; 1 g $L^{-1}$ $MgSO_4$; 1.5 g $L^{-1}$ $KH_2PO_4$ (Sivakesava et al., 2001).

### 2.4 Hybrid saccharification and fermentation of the whole slurry

The whole slurry was hydrolysed for 24 h under enzymatic saccharification before the beginning of the fermentation stage. At the end of the enzymatic step, pH was adjusted to 6 using NaOH 1 M, agitation speed was set to 200 rpm, and the temperature was decreased to 33.4 °C, while inoculum volume fraction was set to 12.3 % v/v. Environment conditioning took 2 hours before the reactor was properly set up for the yeast inoculation. Therefore, after approximately 26 h, the yeast inoculum was added to the reactor. The fermentation of the slurry was performed for 24 h. The results were assessed in terms of relative ethanol yield $Y_{EtOh}$ expressed according to Eq(3):

$$Y_{EtOh} = \frac{C_{EtOH}(t = 33h) - C^0_{EtOH}}{0.511\ (C^0_S - C_S(t = 33h))}\ 100 \quad (3)$$

Where, $C_{EtOH}(t = 33h)$ is the ethanol concentration obtained after 33 hours from the beginning of the experimental run, $C^0_{EtOH}$ is the ethanol concentration recorded immediately after the inoculation. $C_S(t = 33h)$ and $C^0_S$ are the sum of glucose, xylose and arabinose concentrations related to the same sampling times. To allow a comparison with yields reported in the literature, xylose consumption and arabinose consumption were also included in the calculations. The theoretical yield is 0.511. The reference value used to calculate the ethanol yield was obtained 33 h from the beginning of the experimental run (i.e., 7 h after inoculation) due to the observed ethanol consumption resulting from glucose depletion.

### 2.5 Analytical methods

Aliquots of 3 ml of the fermenting slurry (liquid+solid) were sampled over time, filtered through 0.20-µm membranes (Sartorius, Germany) and then analysed by HPLC (Ultimate 3000, Thermo Fisher) equipped with a refractive index detector (Dionex Softron GmbH, Germany) and a Phenomenex Rezex RHM-Monosaccharide H+ (8%) analysis column working at 79°C with ultrapure water as mobile phase (0.6 mL/min). Glucose, xylose, arabinose, and ethanol were quantified through calibration curves. The limits of the curve were [0.1–50 g/L] for glucose, [1–10 g/L] for xylose, [1–10 g/L] for arabinose, with $R^2_{Glucose} = 0.9993$, $R^2_{Xylose} = 0.9917$ $R^2_{Arabinose} = 0.9997$.

## 3. Results

The first step of the investigation concerns the assessment of the enzymatic hydrolysis performance under high solid loadings in order to evaluate the extent of glucose released in such a mass transfer-controlled system. Afterwards, the fermentation stage of pre-hydrolysed brewer spent grain was assessed by means of ethanol yield relative to its theoretical value. Finally, a comparison with literature values is reported for both processes.

### 3.1 Enzymatic hydrolysis of raw BSG

Before the yeast inoculation, raw BSG was pre-hydrolysed to reduce the system's viscosity and guarantee higher fermentation rates after inoculation. Figure (1.a) shows the time profile of glucose, xylose and arabinose during the saccharification. It is worth noticing that the initial glucose concentration is different from zero because of the amount introduced with the enzyme (4.5 g/L) and the amount contained in SGP (1.49 g/L), giving a total initial glucose concentration of approximately 6 g/L. In order to show only the glucose produced by saccharification, Figures (1.a) reports the difference between the measured glucose concentration and its initial value. Both glucose and xylose concentrations reached the steady state value after 21 hours, while arabinose content was still slightly increasing. This may be due to the difference in affinity of hemicellulase for xylose and arabinose. Indeed, it can be seen that after 24 hours, xylose and arabinose yield (calculated using the same equation for glucose without the enzyme contribution) differs by 4% (Figure1.b). The higher glucose production with respect to the other sugars may be due to a greater cellulase enzyme content in the enzyme blend. Nevertheless, this paper focuses on glucose concentration because the yeast used in this work is highly selective toward glucose. Therefore, this aspect was not further investigated. Regarding hexose sugar production, 33% glucose yield was obtained after 24 h of enzymatic hydrolysis. A similar value was obtained in the literature at similar operative conditions (Michelin and Texeira, 2016). Overall, 26% of potential hexose and pentose sugars in raw BSG were experimentally obtained by enzymatic saccharification.
The sugar concentrations did not significantly change during the environment conditioning, but a slight decrease occurred due to dilution after the inoculation.

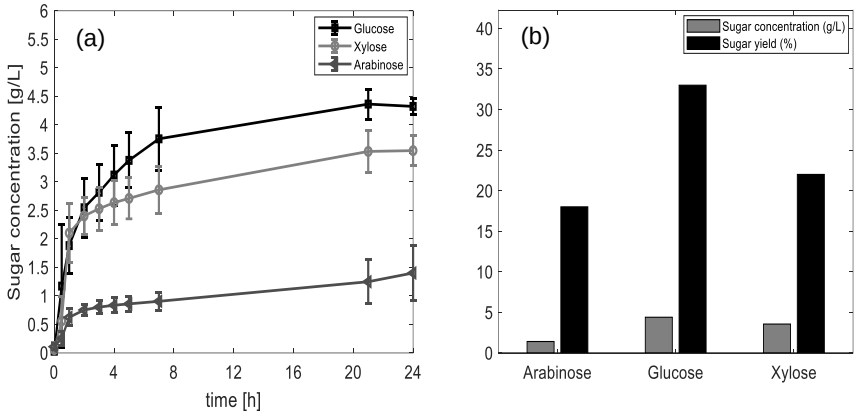


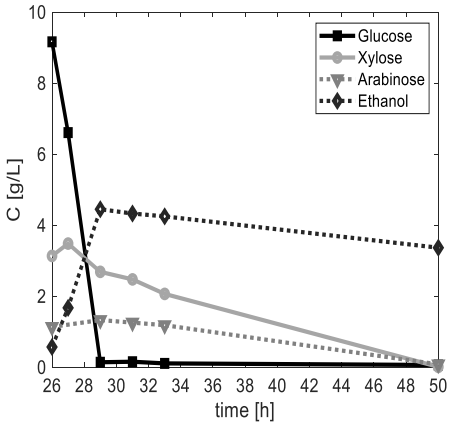

*Figure 1: Sugar vs time profile for the enzymatic hydrolysis carried out in duplicate (a). Glucose concentration values were reported as deviations from the initial value. Comparison of sugar concentration and sugar yield obtained after 24 h of raw BSG saccharification (b).*

### 3.2 Hybrid saccharification and fermentation of the whole slurry

Once the raw BSG was pre-hydrolysed, the resulting broth was fermented by Saccharomyces Cerevisiae. The inoculum loading of 12.3 % (v/v) was introduced after 26 h (two of which were required for system conditioning). Figure 2 shows how the sugars and ethanol concentration evolved during the whole fermentation step. It is important to specify that the contribution of glucose content in the enzymatic blend and the SGP is being considered in this analysis because microorganisms also consume that amount of glucose. The plot reported in Figure 2 underlines how fast is glucose depletion under microbial fermentation. During the first hour after inoculation, xylose and arabinose were still produced by the enzymatic saccharification, and the microorganism consumed 28% of the glucose present at the inoculation. After the first hour of fermentation, the yeast also started to consume xylose, while it took 3 hours for the microorganism to start consuming the arabinose to an appreciable extent. Once the glucose was depleted entirely (after 3 hours), consumption of pentose sugars was more noticeable, and ethanol production slowed, highlighting the scarce capability of *S. Cerevisiae* to convert xylose and arabinose into ethanol, which is widely reported (Rojas-Chamorro et al., 2020). Finally, it was found that after 7 hours, the ethanol yield with respect to the theoretical value was 72%, which complies with the

results obtained in other works (Pinheiro et al., 2019), where *S. Cerevisiae* fermented pre-treated BSG by high temperature autohydrolysis. Similar results were shown in other papers, but only a few are reported here for brevity (Table 2).

*Figure 2. Fermentation of enzymatic hydrolysate: sugars and ethanol time profile after inoculation of S. Cerevisiae on the enzymatic hydrolysate.*




Once the glucose was entirely depleted (after 3 hours), *S. Cerevisiae* started to consume ethanol as a source of carbon, leading to a slight reduction of its concentration. After 24 hours, all the sugars were completely consumed, and the only carbon source (among the ones described) the cells were able to consume was ethanol, leading to a reduction equal to 24% of its maximum value (4.45 g/L), corresponding to the third hour of SSF. One should highlight that, for this process, the microorganism had completely depleted the pentose sugars after 24 hours. We compared the fermentation performances obtained in this study with other studies in terms of ethanol yield. It can be underlined that the same biomass, pre-treated under different conditions, has shown similar results.

*Table 2: Ethanol yield obtained by fermentation of differently pre-treated BSG by means of S. Cerevisiae.*

| Pretreatment | Solid Loading | Ethanol Yield (%) | Reference |
|---|---|---|---|
| Untreated | 6.3 % (w/w) | 72 | This study |
| Phosphoric acid[b] | 5 % (w/v) | 72 | Rojas-Chamorro et al., 2017 |
| Sulphuric acid[a,b] | 5 % (w/v) | 68 | Rojas-Chamorro et al., 2020 |
| HCl catalysed microwave[b] | 30 %(w/v) | 73 | Wilkinson et al., 2015 |
| Autohydrolysis[b] | 30 % (w/v) | 75 | Wilkinson et al., 2015 |
| Autohydrolysis | 25% (w/v) | 73 | Pinheiro et al., 2019 |

[a] Cofermentation with *Scheffersomyces Stipitis*
[b] Only solid residue was enzymatically hydrolysed

Interestingly, the cited results were obtained by fermenting a near inhibitor-free substrate since Wilkinson et al. (2015) washed the pre-treated biomass, Rojas-Chamorro et al. (2020) detoxified acid pre-treated BSG before the enzymatic saccharification, while Pinheiro et al. (2019) reported a negligible concentration of inhibitory compounds downstream the autohydrolysis pretreatment. This may suggest that the mild condition at which raw BSG was hydrolysed in this work allows for reaching good ethanol yields due to low inhibitory compound production.

## 4. Conclusions

The bioconversion process of BSG is a promising option to give added value to lignocellulosic biomass. This study investigated the hybrid saccharification and fermentation configuration to produce ethanol from glucose obtained by enzymatic saccharification of untreated brewer's spent grain. In the enzymatic pre-hydrolysis, the relatively low glucose yield (33%) indicated that mass transfer limitation related to high recalcitrance of solid matrix given by lignin fraction strongly affected the performance of the process under high solid loadings.

On the other hand, fermentation of the whole slurry resulted in an ethanol yield equal to 72% reached after 7 hours from the inoculation. Such an ethanol yield value is comparable to those obtained by pre-treated biomass,

preventing the need to remove inhibitory compounds generated during the hydrolysis process. Furthermore, unlike acid/alkali pre-treated biomass, it is not necessary to use great amounts of base or acid to set the slurry into a fermentable environment. Therefore, it is possible to conclude that HSF of untreated BSG can be considered a technically viable and sustainable strategy to produce ethanol.

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**Nomenclature**

$C_{BSG}$ – concentration of dry solid biomass, g $L^{-1}$
$C_{EtOH}$ – Ethanol Concentration, g $L^{-1}$
$C_S$ – Sugar concentration, g $L^{-1}$
t – time, h
$V_{enz}$ – enzyme volume injected in the reactor, mL
$V_R$ – reactor Volume, L
$x_G$ – glucose mass fraction in dried BSG, -
$x_{G,enz}$ – glucose mass fraction in enzyme blend, -
$x_{Gn}$ – glucan mass fraction in dried BSG, -
$Y_G$ – glucose yield, %
$Y_{EtOH}$ – ethanol yield, %
$\rho_{enz}$ – enzyme density, g $mL^{-1}$

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