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Comparison of Fermentation Strategies for Ethanol Production from Pretreated Brewers Spent Grain

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Brewers spent grain (BSG) is an important co-product derived from beer making process with limited practical applications. The bioconversion into ethanol of this renewable resource can be interesting because of its carbohydrate content. Untreated brewers spent grain contains 20.5 % glucans and 25 % hemicellulose, mainly xylose and arabinose. This work focuses on the bioethanol production from this feedstock after phosphoric acid pretreatment at previously determined optimal conditions. The sequential and simultaneous process configurations for saccharification and fermentation were compared at different solid loadings, 5 %, 10 % and 15 % (w/v). In the sequential process, the enzymatic hydrolysis showed good performance. The cellulose saccharification was almost complete even when the enzymatic hydrolysis was performed at the highest solid loading (15 % w/v).
The final ethanol concentrations and yields did not differ significantly between both process configurations tested at 5 % and 10 % solid loading. However, at the highest solid loading the separate process appears to be more favourable. Thus, the best results were achieved when the pretreated BSG was saccharified and fermented by a sequential process with maximum bioethanol production of 22.5 g/L. This ethanol concentration corresponds to a yield of 37 g ethanol/100 g glucose in pretreated BSG and 72 % of the theoretical ethanol yield.

1. Introduction

Brewers' spent grain (BSG) constitutes a byproduct of beer making process yearly generated in big amounts and lack of economic feasible applications. Nowadays, it is mainly used as animal feed due to its high protein content (Mussatto and Roberto, 2005). BSG can be used as raw material for bioethanol production and other added-value products due to its content in carbohydrates, protein and phenols (Xiros and Christakopoulos, 2012). The fractionation of BSG would allow the valorization of the different streams in the biorefinery concept. Other applications for BSG such as biochar production for ammonia-nitrogen removal (Zhang and Wang, 2016) or to replace wood in particleboards (Klímek et al., 2017) have been reported.
The use of mineral acids like sulfuric acid in the pretreatment of lignocellulosic biomass is very common. However, phosphoric acid even though being more expensive, it shows some advantages for being less corrosive as well as for producing lower levels of toxic compounds (Castro et al.,2014). Pretreatment with dilute phosphoric acid has been reported with different lignocellulosic materials such as rapeseed straw (López-Linares et al., 2013), sugarcane bagasse (Castro et al., 2014) or olive tree biomass (Martínez-Patiño et al., 2015). After pretreatment, enzymatic saccharification, fermentation, and product recovery are crucial steps to the bioconversion of lignocellulosic raw material to ethanol. The process simplification by integration of these process steps is essential to reduce the overall production costs. Thus, simultaneous saccharification and fermentation (SSF) offers that important advantage *versus* the separate process (SHF). Moreover, the simultaneous strategy eliminates the end-product inhibition of cellulose hydrolysis. However, the sequential process shows as main advantage the fact that every step can be carried out at its optimal conditions (Saha et al., 2011).
The aim of this work was to compare both process strategies, sequential and simultaneous saccharification and fermentation to produce ethanol from phosphoric acid pretreated BSG. The influence of the solid loading in both

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processes was also evaluated. Substrate concentrations of 5 %, 10 % and 15 % (w/v) were investigated with both process configurations since the production of highly concentrated ethanolic solutions is crucial with regards to an industrial scale.

2. Materials and methods

2.1 Raw material and dilute phosphoric acid pretreatment

Brewers spent grain was kindly supplied by a local brewer in Jaén (Spain) with 78 % moisture. This feedstock was previously water washed until neutral pH and then oven dried at 50 ºC until 10 % moisture approximately. Untreated and acid pretreated BSG were characterized using standard laboratory analytical procedures for biomass analysis provided by the National Renewable Energy Laboratory concerning preparation of samples (Hames et al., 2008), ash content (Sluiter et al., 2008) and structural carbohydrates and lignin (Sluiter et al., 2010). The nitrogen content in raw BSG was determined by an EA1112 Thermo Finnigan Elemental Analyser and it was converted into protein using the N x 6.25 conversion factor.
Dilute phosphoric acid pretreatment of BSG was carried out in a 1 L reactor (Parr Instr. Co., IL, USA). 75 g of dried BSG was suspended in 600 mL of aqueous phosphoric acid solution 2 % (w/v) at 155 ºC. These conditions were previously optimized in terms of hemicellulosic sugar recovery and enzymatic hydrolysis yield. After pretreatment, liquid and solid fractions were separated by vacuum filtration. The solid fraction was washed with distilled water to eliminate acid solution, dried at 35 ºC and analyzed for sugar and lignin content. Several reactors were necessary to obtain enough amount of pretreated solid to allow saccharification and fermentation assays to be conducted using different process strategies at high solid loading.

2.2 Enzymatic Saccharification

The enzymatic hydrolysis based on commercial Cellic® CTec3 (Novozymes A/S, Denmark) was carried out in 100 mL Erlenmeyer flasks. Enzyme loading was 15 FPU/g substrate of Cellic® CTec3 supplemented with β-glucosidase (15 IU/g substrate). Enzymatic saccharification of acid pretreated BSG was performed at 5, 10 and 15 % solids with 0.05 M sodium citrate buffer (pH 4.8). The flasks were incubated at 50 ºC in an orbital shaker (Certomat-R, B-Braun, Germany) at 150 rpm for 72 h. Samples were withdrawn at 24, 48 and 72 h for glucose concentration measurements and compared to commercial cellulose (Sigmacell) controls with the corresponding loading. All experiments were carried out in triplicate, the average results and standard deviations are shown. The concentrations of initial glucose in the commercial enzyme mixtures were also quantified by HPLC and subtracted from the final glucose concentrations by enzymatic hydrolysis.

2.3 Microorganism, medium and yeast cultivation

Saccharomyces cerevisiae (Fermentis Ethanol red, France) was used for fermentation assays. Yeast inoculum was prepared in glucose synthetic media consisting of (g/L): yeast extract, 5; $ClNH_4$, 2; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 0.3; and glucose, 30. Cells were grown on a rotary shaker (Certomat-R, B-Braun, Germany) at 150 rpm and 30 ºC for 24 h.

2.4 SHF experiments

The slurries obtained after enzymatic hydrolysis at different solid loadings (5 %, 10 % and 15 % w/v) were centrifuged at 10,000g (Sigma 1-14 Centrifuge) for 10 min. The resulting supernants (glucose solutions) were submitted to fermentation for 24 h in an orbital shaker at 35 ºC and 150 rpm after adjusting to pH 5 with NaOH. Fermentation tests were performed in 100 mL Erlenmeyer flasks containing 25 mL of fermentation medium (hydrolysates) with the nutrients described above for inoculum cultivation except for glucose, which was replaced for the corresponding sugar solution obtained after every enzymatic hydrolysis. The flasks were equipped with a thick rubber stopper, through which one stainless-steel needle had been inserted to permit evolved CO_2 to leave and maintain microaerobic conditions. Fermentation assays started by inoculation of a cell suspension resulting in an initial cell mass concentration of 0.25 g/L. All experiments were carried out in triplicate and the average results are given. Ethanol and sugar concentrations were measured at the end of fermentation process, at 24 h.

2.5 SSF experiments

The SSF experiments were performed under microaerobic conditions in a total volume of 25 mL in citrate buffer (50 mM, pH 4.8) containing the nutrients described for SHF (Section 2.4) and also using the pretreated solids as substrates. The weigh percentage of pretreated substrates were 5 %, 10 % and 15 % (w/v) based on oven-dried material. SSF was started by adding simultaneously enzymes and *S. cerevisiae* inoculum (4 % v/v) yeast

cultures, corresponding to a cell addition of 0.25 g/L. Enzyme dosages used were the same as in the enzymatic hydrolysis tests (Section 2.2). To improve the performance of enzymatic hydrolysis in the SSF process, a set of experiments was carried out with xylanases at the same dosage of cellulases. The assays were carried out at 40 ºC and compared to controls that contained the same cellulose loading (Sigmacell). Samples were taken at different times, centrifuged at 10,000g for 10 min to determine the glucose consumed and the ethanol produced. All SSF experiments were performed in triplicate and the average results are given.
SSF results are reported as percentage of the theoretical yield considering that all the potential glucose in the pretreated BSG is available for fermentation, and a theoretical fermentation yield of 0.51 g ethanol/g glucose. The quantity of ethanol produced in blanks of the enzymes and microorganism for each solid loading was considered in all cases.

3. Results and discussion

3.1 Composition of raw BSG and phosphoric acid pretreated BSG

The composition of untreated BSG used in this work was as follows (dry weight): 22.5 % glucan; 16.9 % xylan, 1.3 % galactan, 6.8 % arabinan, 0.3 % mannan, 14.4 % lignin, 2.3 % ash, and 21.2 % protein. After phosphoric acid pretreatment (155 ºC, 2 % H_3PO_4, 0 min, 12.5 % solid loading), 36.4 % solids were recovered (acid pretreated BSG) with 35.5 % glucan, 1.8 % xylan and 42 % lignin. Likewise, the liquid fraction from the pretreatment (prehydrolysate) accounted for about 30 g/L sugars, mainly xylose (48 %) and arabinose (29 %). The valorisation of this sugar stream was not the aim of this work.

3.2 Enzymatic saccharification and fermentation

SHF was run at three different solid loadings (5 %, 10 % and 15 %). Enzymatic hydrolysis tests were extended up to 72 h although it is worth noting that more than 90 % glucose had been solubilised at 24 h in all cases (data not shown). As expected, by increasing the substrate concentration in the hydrolysis, sugar solutions more concentrated were obtained. Thus, the highest concentration, 59.4 g/L glucose was achieved by using 15 % solid loading in the enzymatic saccharification (Table 1).

Table 1: Glucose concentrations and yields after 72 h enzymatic hydrolysis. Ethanol concentrations and yields after 24 h fermentation in SHF of brewer's spent grain at different solid loadings.

Solid loading (%, w/v)	Glucose concentration (g/L)	Enzymatic digestibility (%)	Enzymatic hydrolysis yield (%)	Ethanol concentration (g/L)	Ethanol yield (%)
5	19.0 ± 0.74	97.4	62.2	7.3 ± 0.06	36.5 (71.6)
10	39.5 ± 0.63	97.4	62.2	15.1 ± 0.14	37.8 (74.1)
15	59.4 ± 0.64	99.7	63.7	22.6 ± 0.19	36.8 (72.2)

Enzymatic digestibility: g glucose by enzymatic hydrolysis/100 g glucose in pretreated BSG.
Enzymatic hydrolysis yield: g glucose by enzymatic hydrolysis/100 g glucose in untreated BSG.
Ethanol yield: g ethanol/100 g glucose by enzymatic hydrolysis.
Data in parenthesis based on maximum theoretical ethanol yield on available glucose.

According to the values of enzymatic digestibility, the cellulose in pretreated BSG was almost completely hydrolysed regardless the solid loading used in the enzymatic hydrolysis. Moreover, it should be noted that the use of high solid concentration did not mean a drop in the enzymatic hydrolysis yield. Enzymatic hydrolysis with 15 % solid concentration yielded 63.7 g glucose/100 g glucose in original BSG (Table 1). This fact results very advantageous because the generation of concentrated sugar solutions allows to obtain high alcoholic concentrations and it is essential to reduce the cost of the subsequent distillation stage. The enzymatic hydrolysis yields obtained in this study compare favourably with those obtained with the same feedstock pretreated with 1 % HCl hydrolysed also at 15 % solids (Wilkinson et al., 2016) or by un-catalysed steam explosion hydrolysed at 3 % solids (Kemppainen et al., 2016).
In the SHF configuration process, the hydrolysates resulting from enzymatic saccharification at different solid loadings were fermented by *S. cerevisiae.* Ethanolic solutions with concentrations that ranged between 7.3 g/L at 5 % solids and 22.6 g/L at 15 % solids were obtained. Thus, the best results were reached by enzymatic saccharification at 15 % solids since it yielded the highest ethanol concentration maintaining a good ethanol yield, 36.8 g ethanol/100 g glucose in the hydrolysate (Table 1). Similar ethanol yields were achieved from BSG pretreated by microwave also in a separated configuration process (Wilkinson et al., 2015).

3.3 Simultaneous saccharification and fermentation of pretreated BSG

For comparison purposes, acid pretreated BSG was subjected to an SSF process at the same solid loadings than those tested in the sequential process (5 %, 10 % and 15 % w/v). The results showed that glucose released by the enzymes was completely consumed even with the highest solid loading assayed, 15 % (Figure 1). Ethanolic solutions ranging from 7.2 g/L with 5 % solids up to 18.5 g/L with 15 % solids were obtained. As can be seen in Figure 1, in the SSF experiments at 5 % and 10 % solids, the bioconversion had been completed before 48 h. However, when solid loading was as high as 15 %, SSF process required a longer time to achieve the maximum ethanol concentration. Similar ethanol yields were reached at 5 % and 10 % solid loading, 72.4 % and 73.6 % of the theoretical ethanol yield, respectively (Table 2). However, more than 10 % decrease was determined when the SSF process was carried out at 15 % solids (62 % ethanol yield) although glucose accumulation in the medium was not observed. This can be due to the poor performance of enzymatic hydrolysis indicating that part of the cellulose in the acid pretreated BSG remained without hydrolysing after 72 h enzymatic hydrolysis.

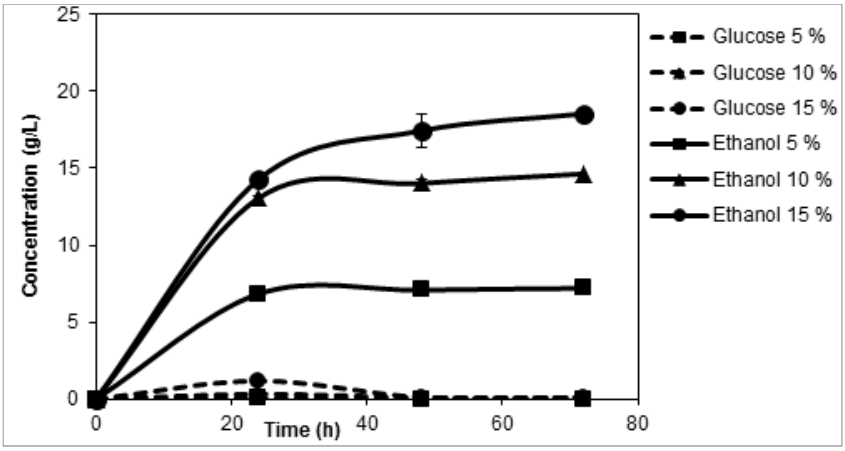

Figure 1: Glucose (dashed lines) and ethanol (continuous lines) concentrations during SSF (without xylanases) of acid pretreated brewer`s spent grain at 5 % (■), 10 % (▲) and 15 % (●) solids loading.

Table 2: Comparison between ethanol production from SHF and SSF with and without xylanases.

Process configuration	Solid loading (w/v)	Ethanol concentration (g/L)	Ethanol yield (%)	*Ethanol productivity (g/L/h)
SHF after 72 h HE + 24 h fermentation	5	7.3 ± 0.06	37.5 (73.5)	0.305
	10	15.1 ± 0.14	38.7 (75.9)	0.629
	15	22.6 ± 0.19	38.6 (75.7)	0.940
SSF without xylanases after 72 h fermentation	5	7.2± 0.11	36.9 (72.4)	0.285
	10	14.6 ± 0.24	37.5 (73.6)	0.544
	15	18.5 ± 1.07	31.7 (62.1)	0.595
SSF with xylanases after 30 h fermentation	5	7.3 ± 0.67	39.8 (78.1)	0.268
	10	14.2 ± 0.30	38.7 (75.9)	0.558
	15	20.7 ± 0.29	37.6 (73.8)	0.829

Ethanol yield referred to glucose in pretreated BSG
Data in parenthesis: based on maximum theoretical ethanol yield on available glucose.
* Ethanol productivities determined at 24 h fermentation time

To overcome this drawback, xylanases were added to the enzyme cocktail used in the previous experiments. In spite of the negligible xylan content in the acid pretreated BSG, according to Hu et al. (2011), a synergistic interaction between the xylanases and the cellulases exits and can result in significant enhance cellulose

accessibility. Figure 2 shows the time course of SSF experiments carried out with addition of xylanases. As shown, glucose was completely converted before 30 h with the three solid loadings tested and the maximal ethanol concentrations were achieved at that time.

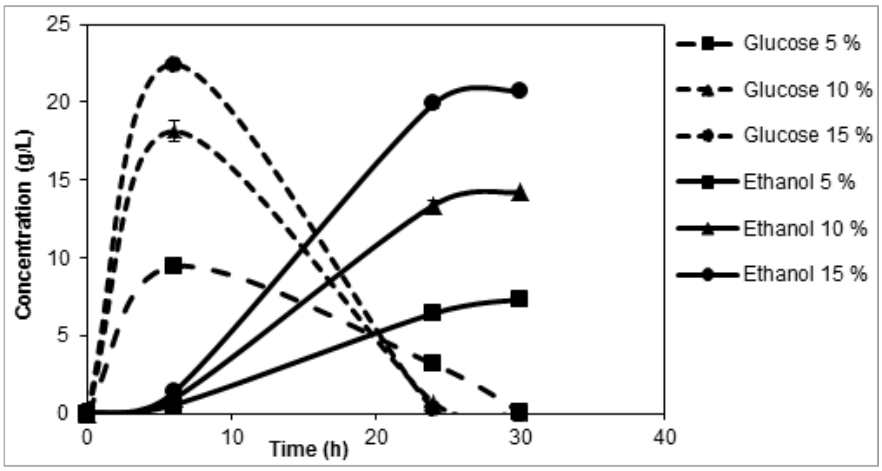

Figure 2: Glucose (dashed lines) and ethanol (continuous lines) concentrations during SSF with xylanases of pretreated brewer`s spent grain at 5 % (■), 10 % (▲) and 15 % (●) solids loading.

As shown in Figures 1 and 2, the use of xylanases improved noticeably the ethanol production at the highest solid loading, 20.7 g/L *vs* 18.5 g/L. Thus, at 15 % solid loading, ethanol yield increased from 31.7 g/g to 37.6 g/g that corresponds to 62.1 % and 73.8 % of the theoretical ethanol yield, respectively. Furthermore, it should be noted that the presence of xylanases in the medium shorted remarkably the process time, mainly at 15 % substrate loading. Obviously, this fact affected to the ethanol productivity, 0.595 g/L/h *vs* 0.829 g/L/h after 24 h fermentation time (Table 2).

In order to know the performance of the enzymatic hydrolysis in the SSF configuration carried out with xylanases, the composition of residual solids was determined with the same analytical methods used for acid pretreated BSG. Thus, residual solids with high lignin concentration were obtained after simultaneous saccharification and fermentation process. Moreover, the presence of cellulose was also detected in all of them (Table 3). As can be expected, maximum cellulose content in the residual solid was detected when SSF process was carried out with the highest solid loading. Thus, after 30 h SSF at 15 % solid loading, 11.2 % cellulose remained without be hydrolysed (Table 3). This fact can be explained because the presence of high substrate concentration in the medium hinders the necessary enzyme-substrate interactions for the enzymatic hydrolysis (Koppram et al., 2014).

Table 3: Composition of residual solids after SSF process with xylanases at different solid loadings

Solid loading (%)	Cellulose (%)	AIL (%)	ASL (%)
5	3.93	72.33	3.09
10	8.43	67.27	2.84
15	11.22	62.42	3.01

AIL: Acid insoluble lignin
ASL: Acid insoluble lignin

Comparing the three studied process configurations for acid pretreated BSG (Table 2), sequential process (SHF) resulted in higher ethanol production that than in the simultaneous process (SSF) even with the use of xylanases. However, this configuration required longer process times to achieve maximal ethanol productions, i.e., 96 h (72 h hydrolysis plus 24 h fermentation) versus 30 h in the SSF process with xylanases.

Concerning the ethanol productivity after 24 h fermentation time, compared with the simultaneous hydrolysis and fermentation, SHF gave higher productivities for the three solid loadings assayed. The highest value of ethanol productivity achieved in this work was in the SHF, 0.94 g/L/h, when the enzymatic hydrolysis was carried out with 15 % acid pretreated BSG.

4. Conclusions

Brewers spent grain is an interesting raw material for ethanol production. Dilute phosphoric acid has been tested as an effective pretreatment for this feedstock yielding an enriched-cellulose substrate highly digestible.
In the SSF process, the results indicated that the use of xylanases shortened the bioconversion time although the ethanol production was only improved at the highest solid loading. By comparing both SSF and SHF configurations, the highest ethanol production was reached with SHF, although it required 96 h (72 h enzymatic hydrolysis plus 24 h fermentation) to complete the bioprocess while SSF (with xylanases) only needed 30 h. Therefore, to balance, we can conclude that the simultaneous process with xylanases of acid-pretreated BSG at 15 % solids can be the best option with ethanol production very close to the maximal one obtained in this work in a much shorter time (21 g/L ethanol, 74% theoretical yield).
Future research should be focused on the saccharification and fermentation of the whole slurry to achieve a cost-effective process by reducing process steps.

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