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TOTAL REUSE OF BREWER'S SPENT GRAIN IN CHEMICAL AND BIOTECHNOLOGICAL PROCESSES FOR THE PRODUCTION OF ADDED-VALUE COMPOUNDS

S.I. Mussatto[1], G. Dragone[2], J.A.Teixeira[3] and I.C. Roberto[4]
[1] IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. E-mail: solange@deb.uminho.pt
[2] IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. E-mail: gdragone@deb.uminho.pt
[3] IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. E-mail: jateixeira@deb.uminho.pt
[4] Department of Biotechnology, Engineering College of Lorena, University of São Paulo, 12607-030, Lorena, SP, Brazil. E-mail: ines@debiq.eel.usp.br

ABSTRACT
Brewer's spent grain was fractionated by means of three different procedures: dilute acid hydrolysis, for the hemicellulose recovery; alkaline hydrolysis, for the lignin solubilization, and enzymatic hydrolysis, for the cellulose conversion into glucose. The best hydrolysis conditions were optimized to each case. The cellulosic and hemicellulosic hydrolysates produced under these conditions were used as fermentation medium for the production of lactic acid and xylitol, respectively. The efficiency of hemicellulose acid hydrolysis was >85% for all the evaluated conditions, but xylitol production was highest (0.70 g/g xylose) when the hydrolysate was obtained at 120 ºC, 17 min, using 1:8 g:g solid:liquid ratio, and 100 mg H_2SO_4/g dry matter. The best alkaline hydrolysis condition (120 ºC, 90 min, 2% w/v NaOH, 1:20 g:g solid:liquid ratio) gave a pulp constituted by 90.4% (w/w) cellulose, and a liquor containing several phenolic acids, mainly ferulic and *p*-coumaric. In the optimum condition of cellulose enzymatic hydrolysis (45 FPU/g dry matter, 100 rpm, 2% w/v substrate, 45 ºC, 96 h), cellulose was converted into glucose with 93.1% efficiency, and lactic acid was produced with high yield (0.98 g/g glucose) from this hydrolysate.

Keywords: brewer's spent grain; cellulose; hemicellulose; lignin; xylitol; lactic acid; phenolic acids

INTRODUCTION
Nowadays, there is great political and social pressure to reduce the pollution arising from industrial activities. Almost all countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Spent grain is the most abundant brewing by-product, representing approximately 85% of total by-products generated [1]. Brewers' spent grain (BSG) is available at low or no cost throughout the year, and is generated in large quantities (20 kg per 100 l of beer produced) not only by large, but also small breweries. Although the large availability, BSG has received little attention as a marketable commodity. Nevertheless, due to its chemical composition rich in carbohydrates and phenolic compounds, it can be of value as raw material in several processes for the production of added-value compounds [2]. The present study shows several alternatives for total reuse of BSG. The material was totally fractioned and the main constituents were used in chemical and biotechnological processes.

MATERIALS AND METHODS
Brewer's spent grain (BSG)
BSG was obtained from a process employing 100% malt (without addition of other cereal adjuncts) and contained (% dry weight) cellulose (16.8), hemicellulose (28.4), lignin (27.8), acetyl groups (1.35), ash (4.6), proteins (15.25) and extractives (5.8). The material, supplied by the microbrewery of the Engineering College of Lorena, was washed with water until neutral pH was achieved, then dried at 50 ± 5 ºC to 10% moisture content and stored until required for processing or analysis.

Acid hydrolysis reactions

Different conditions of solid:liquid ratio (1:8, 1:10 and 1:12 g:g), sulfuric acid concentration (100, 120 and 140 mg/g dry matter) and reaction time (17, 27 and 37 min) were used in the hydrolysis process. Reactions were carried out according to a 2^3 full factorial design, at 120 ºC, in a 1.5 l stainless steel batch reactor, which was filled with 90 g of BSG (90% dry matter) and the required amount of acid solution. After hydrolysis the resulting solid material was separated by filtration and the filtrate (hemicellulosic hydrolysate) was analyzed for determination of sugars (glucose, xylose and arabinose) [3]. Prior to fermentation, the hydrolysate had their pH (1.25) adjusted to 6.5 by addition of NaOH pellets, the precipitate being removed by centrifugation (1100 × *g*, 20min).

Recovered sugar yield Y_S (g of substance that can be obtained from 100 g of BSG dry matter) and hydrolysis efficiency η (%) were calculated using Eq. 1 and Eq. 2, respectively, where C is the concentration of the component in the liquid phase (g/l), M is the amount of BSG (dry matter) employed in the experiment (g), V is the volume of liquid solution used (l) and Y_{max} is the maximum yield of recovered sugars that can be attained (g per 100 g dry matter):

$$Y_S = (C \times V/M) \times 100 \quad (1)$$

$$\eta = (Y_S/Y_{max}) \times 100 \quad (2)$$

Xylitol production

Candida guilliermondii FTI 20037 was the microorganism used for xylitol production. It was grown in 250 ml Erlenmeyer flasks containing 100 ml of the medium (g/l): xylose (20), $(NH_4)_2SO_4$ (3.0), $CaCl_2{\cdot}2H_2O$ (0.1) and 20% (v/v) rice bran extract. The inocula were incubated at 30 ºC in a rotary shaker at 200 rpm for 24 h. Subsequently, the cells were separated by centrifugation (1100 × *g*, 20min) and directly resuspended in the fermentation media. Fermentations were performed in 250 ml Erlenmeyer flasks containing 100 ml of medium (hydrolysates at pH 6.5) and inoculated with an initial cell concentration of 1 g/l. Flasks were agitated at 200 rpm in an orbital shaker at 30 ºC. The fermentation runs lasted 24 h and were monitored through periodic sampling to determine cell growth, glucose and xylose uptakes and xylitol production [3].

Alkaline hydrolysis reactions

The acid pretreated BSG solid residue was submitted to alkaline reactions, which were carried out in 200 ml stainless steel batch cylindrical reactors. Reactions were performed using the BSG and the soda solution in a solid:liquid ratio of 1:20 g:g, at different soda concentrations (1.0, 1.5 and 2.0 % w/v), temperatures (80, 100 and 120 ºC) and reaction times (30, 60 and 90 min), according to a 2^3 full factorial design. Under the required temperature, the dully-covered reactors (filled with the BSG and the soda solution) were introduced into a silicone oil bath, where they were maintained during the desired time. At the end of each reaction, the reactors were immediately cooled in ice bath. The hydrolysate was separated from the pulp by filtration in a 100% polyester cloth [4]. The pulps were washed with water to remove residual alkali, and after drying at 50 ± 5 ºC, a sample of each one of them was analyzed to determine the remaining cellulose, hemicellulose and lignin contents [5]. The resulting liquors were analyzed to determine the concentrations of phenolic compounds present. All the reactions were carried out in duplicate. Under the optimized alkaline hydrolysis conditions, several batches were performed to obtain enough amount of cellulosic pulp to be used in the enzymatic hydrolysis experiments. These batches were carried out in 500 ml stainless steel batch cylindrical reactors that were introduced into a silicone oil bath with appropriate recirculation and temperature controller.

Enzymatic hydrolysis reactions

A commercial cellulase concentrate, Celluclast® 1.5L, produced by *Trichoderma reesei* (Novozymes A/S, Bagsvaerd, Denmark) was used in the experiments as sole enzymatic complex. The cellulolytic activity of concentrate was 74 FPU/ml. For enzymatic hydrolysis experiments, the cellulase concentrate was diluted in 50 mM sodium-citrate buffer (pH 4.8) containing 0.02% w/v sodium azide. Different agitation speeds (100, 150 and 200 rpm), enzyme loadings (5, 25 and 45 FPU/g substrate), and substrate concentrations (2, 5 and 8% w/v) were used according to a 2^3 full factorial design. The experiments were carried out in 125 ml Erlenmeyer flasks containing 25 ml total reaction volume (the buffer-enzyme mixture). After the substrate addition, the flasks were sealed and incubated in a rotary shaker at 45 ºC for 96 h. To follow the hydrolysis, a flask was

withdrawn at different times and the reaction mixture was immediately centrifuged at 4000 rpm for 10 min, to remove solids. The liquid phase (hydrolysate) was heated for 5 min on a boiling water bath to precipitate the protein and prevent further hydrolysis [6]. The cellulose conversion (CC, as glucose yield and cellobiose yield) was calculated according to Eq. 3.

$$CC\ (\%) = (\text{glucose}/\text{cellulose}) \times 0.9 \times 100 + (\text{cellobiose}/\text{cellulose}) \times 0.95 \times 100 \quad (3)$$

Lactic acid production

Lactobacillus delbrueckii UFV H2B20 obtained from Federal University of Viçosa (Viçosa, Brazil) was the microorganism used in the experiments. Stock cultures were maintained at 5 ºC in test tubes containing MRS agar. The inoculum was prepared by transferring a loopful of cells to 25 ml test tubes containing 10 ml sterile MRS broth (the same composition of MRS agar, without agar-agar). The tubes were statically incubated for 24 h at 37 ºC. Two milliliters of this culture was then transferred to a 250 ml Erlenmeyer flasks containing 110 ml MRS broth, and incubated at the same conditions. Finally, the cells were harvested by centrifugation (1100 × *g*, 15 min) and directly resuspended in the fermentation medium to obtain a cell concentration of 1.0 g/l at the beginning of the fermentations. The hydrolysate was pH adjusted to 6.0 by addition of NaOH 5N. Subsequently, 100 ml of the hydrolysate were transferred to 250 ml Erlenmeyer flasks and sterilized at 112 ºC for 15 min. Assays were performed with pH control at 6.0 by addition of 5N NaOH during fermentation. The flasks were statically incubated at 37 ºC for 60 h [7]. The fermentation performance was evaluated by periodic sampling (2 ml) for determination of the pH, and lactic acid, glucose and cell concentration. The fermentation assays were performed in triplicate.

Analytical procedures

In the acid hydrolysis and xylitol production stages, concentrations of glucose, xylose, arabinose, and xylitol were determined by high-performance liquid chromatography (HPLC) [3]. Glucose, cellobiose and lactic acid concentrations during the enzymatic hydrolysis and lactic acid production stages [7], and the phenolic acids concentrations were also determined by HPLC [8]. Statistica 5.0 was the commercial software employed for regression and graphical analyses of the results.

RESULTS AND DISCUSSION

Acid hydrolysis and xylitol production

The hydrolysis efficiencies of xylan into xylose, and arabinan into arabinose were higher than 85.8% for all the experiments. As a consequence, the hemicellulose hydrolysis efficiency (as xylan + arabinan) was also elevated for all the conditions evaluated (higher than 88.7% -Table 1). According to the statistical analysis, there was no significant difference among the treatments at $p>0.05$.

Table 1. Efficiency of BSG acid hydrolysis under different operational conditions [3].

EXPERIMENT	VARIABLES LEVELS			HYDROLYSIS EFFICIENCY (%)		
	Solid:liquid ratio (g/g)	Acid (mg/g)	Reaction time (min)	Xylan	Arabinan	Hemicellulose
1	1:8	100	17	88.7	100.0	92.7
2	1:12	100	17	87.1	99.0	91.8
3	1:8	140	17	86.7	96.1	89.5
4	1:12	140	17	91.7	100	95.1
5	1:8	100	37	87.2	96.6	90.0
6	1:12	100	37	87.5	97.6	90.5
7	1:8	140	37	91.5	100.0	94.1
8	1:12	140	37	94.2	100.0	96.5
9	1:10	120	27	92.0	100.0	95.3
10	1:10	120	27	89.6	100.0	92.7
11	1:10	120	27	89.1	98.1	91.8
12	1:10	120	27	85.8	95.7	88.7

However, the hydrolysis efficiency is not the unique response that should be considered when studying hydrolysates. For practical applications, their fermentability is also very important and must be evaluated. *C. guilliermondii* was able to grow and produce xylitol in all the hydrolysates, but the fermentation results varied for each medium as a function of the employed hydrolysis conditions (Table 2). Cell growth was almost the same in all hydrolysates, but the xylitol production significantly varied for each one (from 5.32 to 10.76 g/l). As xylitol production tends to increase with the increase of initial xylose concentration [9] these results must be compared in terms of the fermentative parameters ($Y_{P/S}$ and Q_P).

Table 2. Effect of hydrolysis conditions of brewer's spent grain on xylose yield, cell growth, xylitol production, and fermentative process parameters ($Y_{P/S}$ and Q_P) [3].

Experiment	Variables levels			Fermentation results					
	Sol:liq ratio (g:g)	Acid (mg/g)	Reaction time (min)	Initial xylose (g/l)	Xylose consumed (%)	Cells (g/l)	Xylitol (g/l)	$Y_{P/S}$ (g/g)	Q_P (g/l.h)
1	1:8	100	17	21.88	78.7	3.86	10.76	0.70	0.45
2	1:12	100	17	14.28	89.3	3.97	6.30	0.46	0.26
3	1:8	140	17	21.44	75.2	3.65	9.28	0.53	0.39
4	1:12	140	17	15.11	86.1	3.61	6.98	0.50	0.29
5	1:8	100	37	20.96	82.5	4.15	9.07	0.50	0.38
6	1:12	100	37	14.36	94.5	4.51	5.32	0.38	0.22
7	1:8	140	37	22.62	67.0	3.18	9.08	0.55	0.38
8	1:12	140	37	15.48	87.2	3.98	6.43	0.43	0.27
9	1:10	120	27	18.16	79.1	3.27	7.87	0.55	0.33
10	1:10	120	27	17.69	88.1	4.46	8.05	0.51	0.33
11	1:10	120	27	17.60	88.1	4.28	7.76	0.48	0.32
12	1:10	120	27	16.94	96.9	4.57	7.45	0.41	0.31

$Y_{P/S}$ (g/g) = xylitol yield factor (ratio between gram of xylitol produced and gram of xylose consumed at the end of each fermentation); Qp (g/l.h) = xylitol volumetric productivity (ratio between concentration of xylitol produced at the end of each run and the fermentation time).

A statistical analysis was carried out to evaluate the effect of the operational variables on the fermentative parameters values. The solid:liquid ratio was the variable with higher influence on both, $Y_{P/S}$ and Q_P, presenting a negative effect. The contact time also presented statistical significance at 90% confidence level for Q_P, with a negative effect. This means that the fermentation results were favored when *C. guilliermondii* was cultivated in hydrolysates produced at the lowest solid:liquid ratio (1:8 g:g) and reaction time (17 min). The acid concentration presented no main significant effect for these parameters, but its interactions with the solid:liquid ratio and with the reaction time were significant at 90% confidence level for Q_P. The positive signal of these interactions suggests that Q_P was favored in hydrolysates produced with the lowest values of solid:liquid ratio, acid concentration, and reaction time. Based on the statistical analysis results, the better condition for BSG acid hydrolysis with sulfuric acid was established with the use of 1:8 (g:g) solid:liquid ratio, 100 mg H_2SO_4/gram dry matter and reaction time of 17 min [3].

Alkaline hydrolysis and phenolic compounds obtainment

An experimental design was used to evaluate the effect of the operational variables in the BSG alkaline hydrolysis. Table 3 gives the chemical composition of the BSG pulps obtained for each evaluated condition. It can be noted that the cellulose content in the pulps strongly varied to each condition employed (from 43.6%, assay 5, to 72.1%, assay 8), and although most of the treatments promoted insignificant losses in the content of this fraction (< 1.8% for 7 of the 11 assays), cellulose losses up to 23.3% were observed during the alkaline treatment. Table 3 also shows that all the pulps contained a residual hemicellulose, the percentages varying from 5.6 to 10.7% (w/w).

An analysis of the estimated effects of the variables showed a negative and main effect of temperature (p<0.05), reaction time (p<0.05) and soda concentration (p<0.10) on the residual lignin content in the BSG pulp. The negative effects indicate that under the mildest reaction conditions, a

major amount of residual lignin is obtained in the pulp. In other words, less residual lignin is obtained in the BSG pulp when the temperature, reaction time and soda concentration are increased. The cellulose content in the pulps was also strongly influenced by the three variables studied (p<0.01 for all of them). Among these, the temperature presented the highest effect, followed by the soda concentration and reaction time, respectively [4].

Table 3. Experimental matrix for the BSG composition, and cellulose and lignin losses after the alkaline hydrolysis, according to a 2^3 full factorial design [4].

Assay	Variables levels [a]			BSG composition (% w/w)				Losses during hydrolysis (% w/w)	
	X_1	X_2	X_3	Cellulose	Hemicellulose	Lignin	Others [b]	Cellulose	Lignin
1	1.0	80	30	51.2	10.7	28.5	9.6	0	60.5
2	2.0	80	30	54.1	10.0	26.3	9.6	0	65.0
3	1.0	120	30	57.9	7.1	23.8	11.2	10.9	74.7
4	2.0	120	30	55.3	6.1	21.5	17.1	12.5	76.5
5	1.0	80	90	43.6	7.1	22.5	26.8	23.3	72.6
6	2.0	80	90	56.0	8.6	23.5	11.9	1.7	71.5
7	1.0	120	90	61.3	5.6	21.1	12.0	15.0	79.8
8	2.0	120	90	72.1	7.8	10.4	9.7	1.8	90.2
9	1.5	100	60	56.4	6.1	20.1	17.4	0	74.8
10	1.5	100	60	54.9	7.0	21.7	16.4	0	72.3
11	1.5	100	60	55.4	7.2	22.8	14.6	0	71.3

[a] X_1) soda concentration (% w/v); X_2) temperature (ºC); X_3) reaction time (min). [b] other materials include ash, protein and extractives; [c] values correspondent to the mass recovered from 100 g of pretreated BSG.

When the pre-treated BSG was hydrolyzed in a larger volume reactor (500 ml) introduced in a silicone oil bath with appropriate recirculation and temperature controller, a pulp with higher cellulose content was obtained (90.4% w/w), probably due to the best control of the operational conditions used. This pulp was subsequently used in the enzymatic assays.

Besides the cellulose pulp, alkaline hydrolysis of the acid pretreated BSG also resulted in liquors containing phenolic acids, mainly ferulic and *p*-coumaric. Syringic, vanillic, and *p*-hydroxybenzoic acids were found in small amounts in the liquors (Table 4), which also contained vanillin (varying from 3.7 to 8.4 mg/l). Ferulic and *p*-coumaric acids concentration varied according to experimental condition used. The highest concentrations (145.3 mg/l ferulic acid and 138.8 mg/l *p*-coumaric acid) were obtained using the highest soda concentration, temperature and reaction time (assay 8) [8].

Table 4. Experimental matrix for the concentration of phenolic acids in the liquors obtained from BSG alkaline hydrolysis, according to a 2^3 full factorial design [8].

Assay	Variables levels [a]			Concentration in the liquor (mg/l)				
	X_1	X_2	X_3	ferulic acid	*p*-coumaric acid	syringic acid	vanillic acid	*p*-hydroxy-benzoic acid
1	1.0	80	30	74.20	67.10	4.90	3.10	1.30
2	2.0	80	30	85.00	100.70	4.70	2.70	1.50
3	1.0	120	30	85.70	95.80	5.20	3.00	4.50
4	2.0	120	30	111.90	114.70	5.40	2.90	7.80
5	1.0	80	90	91.50	91.60	5.00	2.90	1.60
6	2.0	80	90	100.30	105.90	5.40	2.40	1.40
7	1.0	120	90	112.40	106.70	7.10	2.60	13.70
8	2.0	120	90	145.30	138.80	8.10	3.10	26.90
9	1.5	100	60	99.50	114.40	5.80	3.60	5.50
10	1.5	100	60	92.90	104.10	5.50	3.40	3.60
11	1.5	100	60	96.80	100.80	5.70	3.40	3.10

[a] X_1) soda concentration (% w/v); X_2) temperature (ºC); X_3) reaction time (min).

Enzymatic hydrolysis and lactic acid production

Glucose yield and cellulose conversion during enzymatic hydrolysis of BSG pulp were dependent on the hydrolysis conditions employed. The highest values (93.1% and 99.4%, respectively) were obtained using the lowest agitation (100 rpm) and substrate concentration (2% w/v), and the highest enzyme loading (45 FPU/g), conditions of the run 3 (Table 5) [6]. The hydrolysate obtained under this condition (51 g/l glucose) was used as fermentation medium for lactic acid production by *Lactobacillus delbrueckii*, which produced lactic acid with high yield (0.98 g/g glucose), similar to the maximum theoretical value (1 g/g) [7].

Table 5. Experimental conditions for enzymatic hydrolysis of BSG cellulose according to a 2^3 full factorial design, glucose yield and cellulose conversion to each evaluated condition [6].

Run	Real values			Responses	
	Agitation speed (rpm)	Enzyme loading (FPU/g)	Substrate concentration (% w/v)	Glucose yield (%)	Cellulose conversion (%)
1	100	5	2	42.6	66.0
2	200	5	2	23.7	51.6
3	100	45	2	93.1	99.4
4	200	45	2	84.5	89.1
5	100	5	8	25.9	40.7
6	200	5	8	17.9	37.0
7	100	45	8	73.8	83.8
8	200	45	8	71.2	80.5
9	150	25	5	70.3	79.6
10	150	25	5	67.0	80.5
11	150	25	5	71.7	81.8
12	150	25	5	71.3	81.0

CONCLUSIONS

BSG is an agro-industrial by product of great potential to be used as raw material for the production of added-value products such as xylitol, lactic acid and phenolic compounds. The total reuse of this by-product is not only interesting from an economic point of view, but also environmentally, since the elimination of industrial by-products represents a solution to pollution problems.

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