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The effect of agitation speed, enzyme loading and substrate concentration on enzymatic hydrolysis of cellulose from brewer's spent grain

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Abstract Brewer's spent grain components (cellulose, hemicellulose and lignin) were fractionated in a two-step chemical pretreatment process using dilute sulfuric acid and sodium hydroxide solutions. The cellulose pulp produced was hydrolyzed with a cellulolytic complex, Celluclast 1.5 L, at 45 °C to convert the cellulose into glucose. Several conditions were examined: agitation speed (100, 150 and 200 rpm), enzyme loading (5, 25 and 45 FPU/g substrate), and substrate concentration (2, 5 and 8% w/v), according to a 2^3 full factorial design aiming to maximize the glucose yield. The obtained results were interpreted by analysis of variance and response surface methodology. The optimal conditions for enzymatic hydrolysis of brewer's spent grain were identified as 100 rpm, 45 FPU/g and 2% w/v substrate. Under these conditions, a glucose yield of 93.1% and a cellulose conversion (into glucose and cellobiose) of 99.4% was achieved. The easiness of glucose release from BSG makes this substrate a raw material with great potential to be used in bioconversion processes.

Keywords Brewer's spent grain · Cellobiose · Cellulase · Cellulose · Enzymatic hydrolysis · Glucose

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Introduction

Nowadays, there is a great interest in using lignocellulosic raw materials as substrate for the production of ethanol or other value-added compounds. Various lignocellulosic biomasses, including wood, agricultural and agro-industrial residues can be used for this purpose. Brewer's spent grain (BSG), a brewery lignocellulosic by-product, has recently received considerable attention since it is cheap, available in large quantities and has high carbohydrate content (Mussatto et al. 2006a). However, until now it has been few utilized as raw material in bioconversion processes.

The lignocellulosic biomass is a complex structure mainly constituted by three polymeric fractions, namely cellulose, hemicellulose and lignin. The cellulose (a linear homopolymer of repeated β-D-glucose units linked by glycosidic linkages) is associated with hemicellulose and other structural components, and is also surrounded by lignin sheath. This substrate around the cellulose micro fibrils is partly covalently associated with hemicellulose, thus

covering some region of cellulose (Varga et al. 2002). Therefore, the manufacture of chemicals from lignocellulosic biomass requires, in a first step, the material fractionation for separation of its main polymeric constituents.

Chemical and enzymatic methods are the most common techniques for hydrolyzing cellulose. In the chemical method, the hydrolysis is catalyzed by an acid; whereas in the enzymatic method, cellulolytic enzymes act as catalysts (Martín et al. 2002). Currently, enzymatic hydrolysis has attracted increasing attention as an alternative to acid hydrolysis because the process is highly specific, can be performed under milder reaction conditions (pH around 5 and temperature less than 50 °C) with lower energy consumption and lower environmental impact. In addition, it does not present corrosion problems, and gives high yield of pure glucose with low formation of by-products that is favorable for the hydrolysate use in bioconversion processes (Frennesson et al. 1985; Liao et al. 2005; Martín et al. 2002; Wen et al. 2004). Due to these advantages, the enzymatic hydrolysis has been extensively studied in several lignocellulosic substrates, including rice straw (Kaur et al. 1998; Vlasenko et al. 1997), rice hull (Sharma et al. 2001), corn stover (Varga et al. 2002), corncob (Chen et al. 2007), dairy manure (Liao et al. 2005), softwoods (Pan et al. 2005; Wu et al. 1999), and sugarcane bagasse (Martín et al. 2002).

According to the literature, lignocellulosic materials must be pretreated prior to the enzymatic hydrolysis in order to make the cellulose more accessible to enzymes (Berlin et al. 2005; Martín et al. 2002; Varga et al. 2002). Furthermore, the efficient use of enzymes depends on process variables such as temperature, pH, reaction time, enzyme concentration, pulp consistency (substrate concentration), intensity of agitation, and presence of other chemical species that may inhibit or accelerate their rates of reaction (Kaya et al. 2000). Consequently, for the enzymatic process be performed with high efficiency, an optimization of the hydrolysis conditions is required. The present work evaluated the effect of agitation speed, enzyme loading and substrate concentration on enzymatic hydrolysis of cellulose from BSG. The assays were performed according to a 2^3 full factorial design in an attempt to establish the optimal conditions for maximum glucose yield.

Materials and methods

Raw material pretreatment and preparation of cellulose pulp

The BSG used in this study was provided by the microbrewery of the Engineering College of Lorena. As soon as obtained, the material was washed with water until neutral pH, dried at 50 ± 5 °C to 10% moisture content, and then pretreated by dilute sulfuric acid and subsequently by sodium hydroxide solutions, to remove the protective hemicellulose-lignin wrapper. The used reaction conditions were previously optimized and consisted in the use of: 1.25% w/v H_2SO_4 solution in a 1:8 g:g solid:liquid ratio, 120 °C for 17 min (Mussatto and Roberto 2005); and 2% w/v NaOH solution in a 1:20 g:g solid:liquid ratio, 120 °C for 90 min (Mussatto et al. 2006b). After each reaction the residual solid material was separated and washed with water (until neutral pH) to be used in the next step. After washing, the solid fraction resulting from alkaline pretreatment (cellulose pulp) was dried at 50 ± 5 °C to attain 10% moisture content, screened, and only the particles that passed through a 12-mesh standard sieve Tyler serie n° 10 (1.68 mm opening) and were retained in a 18-mesh sieve (1.00 mm opening) were assayed as substrate for enzymatic hydrolysis. The average chemical composition of starting BSG, acid pretreated, and cellulose pulp (expressed in oven-dried basis), are given in Table 1, which also summarizes the product yield after each stage of the processing in dilute acid and base.

Enzyme

A commercial cellulase concentrate, Celluclast® 1.5L, produced by *Trichoderma reesei* (kindly supplied by Novozymes A/S, Bagsvaerd, Denmark) was used in the experiments as sole enzymatic complex. This preparation was a brownish liquid with a density of 1.20 g/mL and contained 27 mg protein/mL. The cellulolytic activity of concentrate was 74 FPU/mL.

Enzymatic hydrolysis

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

Table 1 Chemical composition of brewer's spent grain (BSG) in the starting form, acid pretreated, and cellulose pulp; and product yield after each stage of the processing in dilute acid and base[a]

Component	BSG composition (% dry weight)			Product yield[b] (% dry weight)	
	Starting form	Acid pretreated	Cellulose pulp	After dilute acid processing	After base processing
Cellulose	16.8	34.0	90.4	98.2	96.4
Hemicellulose	28.4	7.9	1.1	13.4	6.2
Lignin	27.8	49.2	8.1	86.0	8.4
Others[c]	27.0	8.9	0.4	16.3	8.2

[a] From Mussatto et al. (in press) and Mussatto et al. (2006b)

[b] Percentage of each fraction recovered after treatment. The values after base processing are correspondent to the percentage recovered from the original starting material

[c] Other components include ashes, protein and extractives

to inhibit microbial contamination. Different agitation speeds, enzyme loadings and substrate concentrations were used according to the 2^3 full factorial design given in Table 2. 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 with a plastic film and incubated in a rotary shaker at 45 °C for 96 h. To follow the hydrolysis, a flask was withdrawn at different times and the reaction mixture was immediately centrifuged at 4,000 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.

Analysis

The chemical composition (cellulose, hemicellulose and lignin) in the starting BSG, acid pretreated, and cellulose pulp was determined as previously described (Mussatto and Roberto 2006). The material was subjected to a quantitative acid hydrolysis with 72% (w/w) sulfuric acid, and the monosacharides contained

Table 2 Experimental conditions for enzymatic hydrolysis of cellulose from brewer's spent grain according to a 2^3 full factorial design, glucose yield and cellulose conversion to each evaluated condition

Run	Variables						Responses	
	Coded levels			Real values				
	Agitation speed	Enzyme loading	Substrate concentration	Agitation speed (rpm)	Enzyme loading (FPU/g)	Substrate concentration (% w/v)	Glucose yield (%)	Cellulose conversion (%)
1	−1	−1	−1	100	5	2	42.6	66.0
2	+1	−1	−1	200	5	2	23.7	51.6
3	−1	+1	−1	100	45	2	93.1	99.4
4	+1	+1	−1	200	45	2	84.5	89.1
5	−1	−1	+1	100	5	8	25.9	40.7
6	+1	−1	+1	200	5	8	17.9	37.0
7	−1	+1	+1	100	45	8	73.8	83.8
8	+1	+1	+1	200	45	8	71.2	80.5
9	0	0	0	150	25	5	70.3	79.6
10	0	0	0	150	25	5	67.0	80.5
11	0	0	0	150	25	5	71.7	81.8
12	0	0	0	150	25	5	71.3	81.0

in hydrolysates were determined by HPLC in order to estimate (after corrections for stoichiometry and sugar decomposition) the contents of samples in cellulose (as glucan) and hemicellulose (as xylan + arabinan). The acid insoluble residue after hydrolysis was recovered by filtration and considered as Klason lignin. The acid soluble lignin was estimated by ultraviolet spectroscopy at 280 nm after adjustment of the samples pH to 12.0 with NaOH 6.0 M, and subsequent dilution of the resulting solution with distilled water in order to obtain an absorbance reading not exceeding 0.5.

Glucose, cellobiose, xylose and arabinose concentrations were determined by HPLC analyses in a Shimadzu LC-10-AD (Tokyo, Japan) chromatograph equipped with a refractive index detector and a Bio-Rad HPX-87H (300 × 7.8 mm) column (Hercules, CA, USA). The samples were diluted with deionized water, filtered through Sep Pak C_{18} filters (Waters Associate Millipore, Bedford, MA, USA) and thus injected in the chromatograph under the conditions: column temperature of 45 °C, 0.01 N sulfuric acid as mobile phase at a flow rate of 0.6 mL/min, and a injection volume of 20 μL. The concentration of these compounds was calculated using calibration curves obtained from standard solutions. The cellulose conversion (CC, as glucose yield and cellobiose yield) was calculated according to Eq. 1, where 0.90 (162/180) and 0.95 (342/360) are the conversion factors (due to the uptake of one water molecule during hydrolysis) of cellulose to glucose and cellobiose, respectively.

$$\mathrm{CC}(\%) = (\mathrm{glucose/cellulose}) \times 0.9 \times 100 + (\mathrm{cellobiose/cellulose}) \times 0.95 \times 100 \quad (1)$$

Filter paper activity of the commercial cellulase concentrate was measured according to Mandels et al. (1976), and expressed in filter paper units (FPU). One-half milliliter of enzyme solution was added into 1 mL of 0.05 M sodium-citrate buffer (pH 4.8) contained in a strip of 50 mg Whatman n° 1 filter paper. The solution was incubated at 50 °C for 1 h. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid (Miller 1959). One unit of FPU is defined as the amount of enzyme required to liberate 1 μmol of glucose from Whatman n° 1 filter paper per minute at 50 °C. The protein concentration in the cellulase concentrate was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

Experimental design and data analysis

A 2^3 full factorial design with four replicates at the midpoint was used to evaluate the influence of three different variables: agitation speed (x_1), enzyme loading (x_2) and substrate concentration (x_3), in the enzymatic hydrolysis of cellulose from BSG. For statistical analysis, the variables were coded according to Eq. 2, where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the midpoint), and ΔX_i (step change value). The coded levels and real values of the variables are given in Table 2. Low and high factors were coded as −1 and +1; the midpoint was coded as 0.

$$x_i = (X_i - X_0)/\Delta X_i \quad (2)$$

Four assays at the midpoint of the design were carried out to estimate the random error needed for the analysis of variance, as well as to examine the presence of curvature in the response surface. The glucose yield and the cellulose conversion (glucose yield and cellobiose yield) were taken as dependent variables or responses of the experimental design. The results were analyzed using the technique of the Analysis of Variance (ANOVA), and the responses and variables (in coded unit) were correlated by the "Response Surface Analysis" of the Statistica 5.0 software (Statsoft, USA) to obtain the coefficients of Eq. 3.

$$\hat{y}_i = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (3)$$

In Eq. 3, $\hat{y}_i$ represents the response or dependent variable; a_0 is the interception coefficient; x_1, x_2 and x_3 are the coded levels of the three variables (agitation speed, enzyme loading and substrate concentration), and a_1, a_2, a_3, a_{12}, a_{13}, a_{23} are the regression coefficients. The statistical significance of the regression coefficients was determined by Student's *t*-test, and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2.

Results and discussion

Pretreatment and characteristics of the substrate

BSG was initially submitted to chemical pretreatment stages for enhancing its susceptibility to the enzymes attack. It has long been recognized that some form of pretreatment is necessary to achieve reasonable rates and yields in the enzymatic hydrolysis of biomasses. This has generally been attributed to three factors: (1) the crystallinity of cellulose, (2) the lignin-hemicellulose sheath that surrounds the cellulose, and (3) the lack of available surface area for the enzymes attack (Wright 1988). Several different pretreatment methods, including physical and chemical procedures, have been thus evaluated to enhance the hydrolytic degradation rate. Physical pretreatments are considered to be relatively less effective in enhancing the hydrolysis rate when compared to chemical pretreatments (Gharpuray et al. 1983). Moreover, physical treatments such as high-energy radiation and ball milling are reported to be energy-intensive and therefore expensive (Varga et al. 2002). Recently, attention has been focused on chemical treatments. The hemicellulose-lignin sheath can be disrupted by either acidic or basic catalysts (McMillan 1994). In our previous works, dilute acid and sodium hydroxide solutions have been shown to be highly effective in removing the hemicellulose and lignin from BSG with the least loss of cellulose (Mussatto and Roberto 2005; Mussatto et al. 2006b). Therefore, in the present work these two procedures were applied for pretreatment of BSG.

Another point of relevance in enzymatic hydrolysis processes is the substrate particle size. The enzymatic hydrolysis of cellulosic materials is a heterogeneous reaction, with soluble enzymes attaching the cellulose and converting it into soluble sugars. Therefore, the substrate particle size is an important parameter for the hydrolysis because it influences the contact between enzyme and substrate (Gan et al. 2002; Mansfield et al. 1999). Since the reaction rate is proportional to the enzyme amount adsorbed into the cellulose surface, decreasing the particle size a major surface area will be available for the enzymes attack, and consequently, the enzymatic hydrolysis will occur with a higher efficiency (Moriyama and Saida 1986; Sattler et al. 1989). In the present work, pretreated BSG was screened and only particles of size between 1.68 and 1.00 mm were used in the enzymatic assays, to facilitate the contact of the enzymes. In addition, the partial hemicellulose and lignin removal during the pretreatment stage also increases the surface area present in the form of pores, increasing the accessibility to enzymes (Converse et al. 1990; Gharpuray et al. 1983; Mansfield et al. 1999).

Enzymatic hydrolysis of pretreated BSG: statistical analysis

Statistical design has proved efficient for optimization of different hydrolysis processes from lignocellulosic materials (Mussatto and Roberto 2005; Mussatto et al. 2006b; Roberto et al. 2003). Therefore, it was here employed to evaluate the effect of the variables agitation speed, enzyme loading and substrate concentration on the enzymatic hydrolysis of BSG cellulose. The design of experiments used and the experimental results achieved are given in Table 2. Both responses, glucose yield and cellulose conversion, were dependent on the conditions employed for BSG hydrolysis, 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.

To optimize the hydrolysis conditions, the first step is identifying the variables that have the greatest influence on the responses. According to Table 3, only the enzyme loading had a highly significant effect ($p < 0.01$) on both responses, with a positive signal, indicating that glucose yield and cellulose conversion were increased when the enzyme loading was increased. The other two studied variables, agitation speed and substrate concentration, did not present significant effects for glucose yield; but for cellulose conversion, the substrate concentration presented a negative and significant effect ($p < 0.10$) indicating that the lower the substrate concentration used, the higher the cellulose conversion achieved.

The statistical significance of main and interaction effects of the variables was determined by analysis of variance. When this analysis was performed without estimation of the curvature, only the enzyme loading was significant at 1% probability level for glucose yield, with a determination coefficient (R^2) of 0.89. Nevertheless, when this analysis was performed with

Table 3 Effect estimates (EE), standard errors (SE) and level of significance for glucose yield and cellulose conversion during the enzymatic hydrolysis of BSG cellulose, according to a 2^3 full-factorial design

Variables and interactions	Glucose yield			Cellulose conversion		
	EE	SE	*p*	EE	SE	*p*
x_1	−9.53	±8.64	0.32	−7.91	±6.34	0.27
x_2	53.11	±8.64	0.00***	39.40	±6.34	0.00***
x_3	−13.77	±8.64	0.17	−16.05	±6.34	0.05*
x_1x_2	3.90	±8.64	0.67	1.15	±6.34	0.86
x_1x_3	4.23	±8.64	0.65	4.41	±6.34	0.52
x_2x_3	−2.53	±8.64	0.78	3.90	±6.34	0.57

*** Significant at 99% confidence level; * Significant at 90% confidence level

x_1, agitation speed; x_2, enzyme loading; x_3, substrate concentration

estimative of the curvature (Table 4), all the studied variables as well as their interactions were significant at least 95% confidence level, and the determination coefficient was increased to 0.99, suggesting a second-order polynomial equation as more suitable to explain the glucose yield variations as function of the evaluated variables in the studied region. Similar results were obtained for cellulose conversion. The analysis of variance for this response revealed that, for a linear model, only the variables enzyme loading and substrate concentration were significant (at 1 and 10% probability level, respectively), with a determination coefficient (R^2) of 0.90. However, when this analysis was performed with estimative of the curvature (Table 4), all the studied variables as well as their interactions (except the agitation speed and enzyme loading interaction) were significant at least 95% confidence level, and the determination coefficient was increased to 0.99, suggesting that a second-order polynomial equation is also more suitable to represent the cellulose conversion as function of the evaluated variables.

To establish these second-order equations it would be necessary to perform additional assays modifying the variables values, but these assays were not carried out for two reasons: (1) a high glucose yield, 93.1%,

Table 4 Analysis of variance (ANOVA) parameters to the significant effects on the glucose yield and cellulose conversion during the enzymatic hydrolysis of BSG cellulose

Factor	Glucose yield					Cellulose conversion				
	SS	d.f.	MS	F	*p*	SS	d.f.	MS	F	*p*
Curvature	741.93	1	741.93	621.55	0.00***	397.23	1	397.23	389.65	0.00***
x_1	181.45	1	181.45	152.01	0.00***	124.98	1	124.98	122.59	0.00***
x_2	5641.34	1	5641.34	4726.06	0.00***	3104.72	1	3104.72	3045.45	0.00***
x_3	378.95	1	378.95	317.47	0.00***	515.21	1	515.21	505.37	0.00***
x_1x_2	30.42	1	30.42	25.48	0.01***	2.62	1	2.62	2.57	0.18
x_1x_3	35.70	1	35.70	29.91	0.01***	38.81	1	38.81	38.07	0.00**
x_2x_3	12.80	1	12.80	10.73	0.03**	30.42	1	30.42	29.84	0.01***
Error	4.78	4	1.19			4.08	4	1.02		
Total	7027.37	11				4218.06	11			

x_1, agitation speed; x_2, enzyme loading; x_3, substrate concentration; SS, sum of squares; d.f., degrees of freedom; MS, mean of squares

$R^2 = 0.999$ for both, glucose yield and cellulose conversion; *** Significant at 99% confidence level; ** Significant at 95% confidence level.

corresponding to a cellulose conversion of 99.4% was achieved in the experimental design proposed. Thus, the additional assays would not result in a significant increase in the yield values; (2) when the analysis of variance was performed for a linear model, high values of R^2 (>0.89) were achieved, showing a close agreement between experimental results and the theoretical values predicted by the first-order polynomials. Therefore, a multiple regression analysis was performed to fit the first-order polynomial equations to the experimental data points. Glucose yield (y_1) and cellulose conversion (y_2) were correlated as a function of agitation speed (x_1), enzyme loading (x_2) and substrate concentration (x_3) (coded values) resulting in Eqs. 4 and 5, respectively.

$$y_1\,(\%) = 59.65 - 4.76\,x_1 + 26.56x_2 - 6.88x_3 + 1.95x_1x_2 + 2.11x_1x_3 - 1.27x_2x_3 \quad (4)$$
$$(R^2 = 0.89)$$

$$y_2\,(\%) = 72.57 - 3.95x_1 + 19.70x_2 - 8.03x_3 + 0.57x_1x_2 + 2.20x_1x_3 - 1.95x_2x_3 \quad (5)$$
$$(R^2 = 0.90)$$

Three-dimensional response surfaces described by the above-mentioned first-order polynomials were fitted to the experimental data points concerning the glucose yield and cellulose conversion (Fig. 1a, b). These figures show that both responses were well-fitted to a flat surface, and the highest values of glucose yield and cellulose conversion can be attained performing the enzymatic hydrolysis of BSG with 2% w/v substrate concentration and enzyme loading of 45 FPU/g substrate (at 100 rpm agitation speed).

Comparison of cellulose conversion from BSG with other lignocellulosic materials

Agitation speed had a negative effect in the glucose yield and cellulose conversion (Table 3), indicating that both responses were increased when the value of this variable was decreased. Similar influence of the agitation speed has been observed during enzymatic hydrolysis of cellulose from other raw materials. For example, using Avicel and paper pulp, Mukataka et al. (1983) verified that excessively high mixing speeds (>200 rpm) lowered the extent of cellulose conversion, while moderate mixing speeds (100–200 rpm)

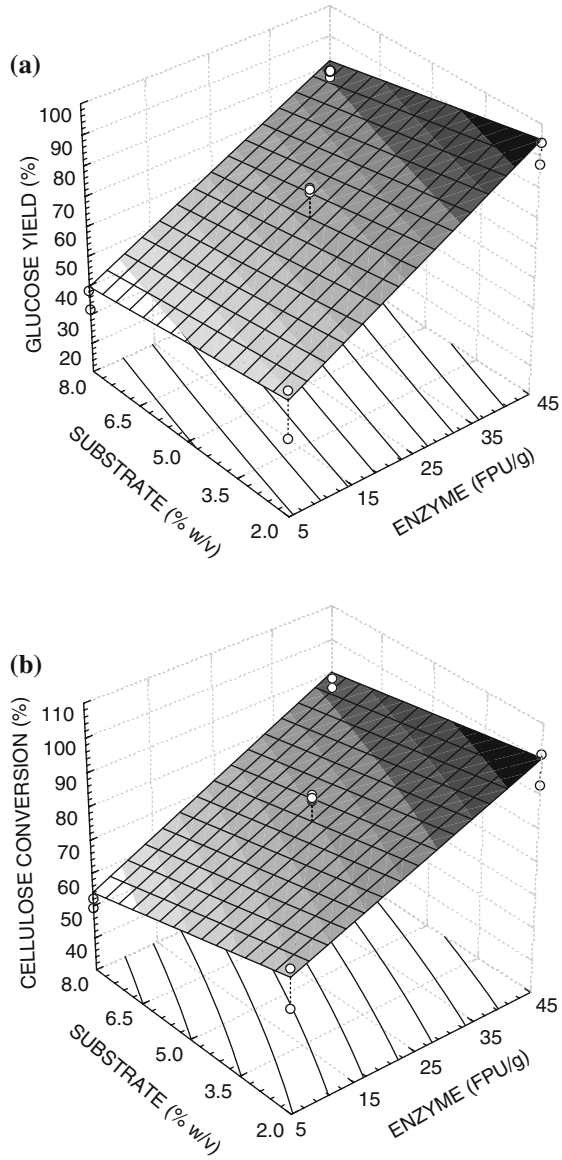

Fig. 1 Response surface fitted to the experimental data points corresponding to (**a**) glucose yield (%) and (**b**) cellulose conversion (%) during the enzymatic hydrolysis of BSG cellulose

provided a good combination of fast initial hydrolysis rates and high conversion yields. Otherwise, increasing the stirring speed from 50 to 90 rpm had insignificant effect on the reducing sugar production from enzymatic hydrolysis of a hardwood derived cellulosic material (Gan et al. 2003).

According to Ingesson et al. (2001) the cellulose hydrolysis by cellulase enzymes requires an adequate mixing to ensure sufficient contact between the

substrate and enzymes, and to promote heat and mass transfer within the reaction vessel. Some agitation increases the hydrolysis rate and yields, but excessive mixing can deactivate the enzymes and reduce the conversion yield (Ingesson et al. 2001; Wright 1988). The deactivation effect has been attributed to shear force generated by the mixer and the entrapment of air bubbles into the medium at the air-liquid surface. In fact, the shear deactivation effect is a deterring factor in applying a more intensive agitation. However, a more properly designed and more intensive mixing regime should facilitate a better mass transfer inside the reactor, reducing a potentially high local product concentration surrounding the cellulase binding and active sites (catalysis sites), facilitating a more dynamic regime of adsorption, catalysis, desorption, and moving to another binding site (Gan et al. 2003).

The substrate concentration was another variable that influenced the glucose yield and cellulose conversion during the enzymatic hydrolysis of BSG (Table 3), with the values of both responses being increased when the substrate concentration was decreased. A close similar behavior was observed during enzymatic hydrolysis of α-cellulose (Ingesson et al. 2001), where the substrate concentration increases from 2.5 to 7.5% w/v reduced the glucose yield in 14%. In the present work, the substrate concentration increase from 2.0 to 8.0% w/v also reduced the glucose yield in an average of 14%.

The effect of substrate concentration on enzymatic hydrolysis of cellulose has also been evaluated for other lignocellulosic materials, including sugarcane bagasse (Manonmani and Sreekantiah 1987), softwood (Tengborg et al. 2001), animal manure (Wen et al. 2004), and corncob (Chen et al. 2007). For all these cases, as well as in the present work, it was observed that glucose concentration and yield showed an opposite trend, with glucose concentration increasing, and yield decreasing with the substrate concentration increase. Such effect can be attributed to end-product inhibition caused by high concentration of glucose, and mass transfer limitations within the reaction mixture due to the high viscosity of the slurry (Ingesson et al. 2001; Wen et al. 2004). Other factors that can contribute to the low degree of carbohydrate conversion at high substrate concentration, mainly when low enzyme loadings are employed, include the decrease in the reactivity of cellulosic material in the course of hydrolysis, different kinds of enzyme inactivation, and non-specific adsorption of cellulolytic enzymes onto lignin (Vlasenko et al. 1997).

Ingesson et al. (2001) compared the effect of substrate concentration and shaking speed in the enzymatic hydrolysis of α-cellulose, and found that the cellulose conversion was more affected by the substrate concentration rather than the shaking speed. In the present work it was observed a similar result, since the agitation speed decrease from 200 to 100 rpm resulted in an average increase in the glucose yield and cellulose conversion of 9.5 and 7.9%, respectively, while the substrate concentration decrease from 8 to 2% w/v resulted in average increases of 13.8 and 16.1%, respectively.

Enzyme loading also influenced the enzymatic hydrolysis of BSG cellulose (Table 3) so that the more the enzymes, the better the hydrolysis. In fact, the effect of enzyme loading was the highest when compared with the effect of the other two studied variables (agitation speed and substrate concentration) since its increase from 5 to 45 FPU/g resulted in an average increase in the glucose yield and cellulose conversion of 53.1 and 39.4%, respectively. Evaluating a similar range of enzyme loading (5–50 FPU/g) during the enzymatic hydrolysis of cellulose from pretreated poplar wood, Sattler et al. (1989) obtained an increase of 31% in the cellulose conversion yield with the enzyme loading increase.

The enzyme loading increase also favored the enzymatic hydrolysis of cellulose from hardwood (Gan et al. 2003), softwood (Pan et al. 2005), willow (Eklund et al. 1990), sugarcane bagasse (Manonmani and Sreekantiah 1987), rice husks (Yáñez et al. 2006), rice straw (Kaur et al. 1998; Vlasenko et al. 1997), and dairy manure fibers (Liao et al. 2005). Nevertheless, the enzyme dosage required to achieve a complete conversion of cellulose into glucose vary to each raw material. For example, during the enzymatic hydrolysis of willow, the maximum conversion of cellulose to glucose was obtained using an enzyme loading of 11.4 FPU/g. Higher enzyme concentrations did not increase the initial hydrolysis rate nor affect the final glucose yield (Eklund et al. 1990). On the other hand, an enzyme loading of 100 FPU/g was necessary to completely hydrolyze the carbohydrate fraction in the acidified steam explosion pretreated rice straw (Vlasenko et al. 1997). In the present work, 45 FPU/g was an enzyme loading enough to promote a complete cellulose conversion.

Perspectives for hydrolysate use as fermentation medium

The optimal conditions for enzymatic hydrolysis of BSG cellulose consisted in using a substrate concentration of 2% w/v. From this substrate amount, the maximum glucose concentration that could be obtained in the hydrolysate was 20.1 g/L. This concentration value is low if it is desired the hydrolysate use as fermentation medium for the production of ethanol or lactic acid, for example. Consequently, the hydrolysate should be concentrated before use as fermentation medium, and this additional step would increase the overall cost of the process. The direct production of a hydrolysate with higher glucose concentration would be more advantageous, since the concentration step would be avoided. This is possible by increasing the substrate concentration used for enzymatic hydrolysis. In the present work, 8% w/v was the maximum substrate concentration evaluated because higher concentration values negatively affected the mixing of the reaction medium due to the high viscosity of the slurry (data not shown). However, the cellulose conversion into glucose was not maximum (yield of 73.8%) when using 8% w/v of substrate and 45 FPU/g of enzyme loading. In an attempt to improve this result, some assays were thus carried out using 8% w/v of substrate, but increasing the enzyme loading up to 85 FPU/g.

The achieved results revealed that the glucose release from BSG cellulose increased with the enzyme loading increase up to 45 FPU/g substrate, but enzyme loadings higher than 45 FPU/g did not favor even more the cellulose conversion into glucose (Fig. 2). Figure 3 clearly shows that the maximum glucose yield was constant for the enzyme loading values ranging between 45 and 85 FPU/g. These results suggest that the enzyme loading increase favor the cellulose hydrolysis, but until a certain limit above of which the velocity of the reaction is not more altered. Therefore, the assays using 45 up to 85 FPU/g achieved similar glucose yield and cellulose conversion of 74 and 84%, respectively. It is probable that an end-product inhibition occurred in these cases, affecting the enzymes activity and the cellulose conversion into glucose, as a consequence. The cellulolytic enzymes from *T. reesei* are highly sensitive to inhibition by hydrolysis products, glucose

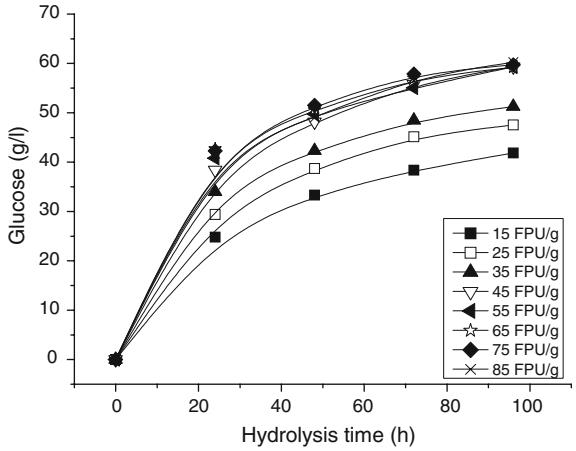

Fig. 2 Glucose release during the enzymatic hydrolysis of the BSG cellulose at 45 °C, 100 rpm, and 8% w/v substrate concentration, using different enzyme loadings (15–85 FPU/g substrate)

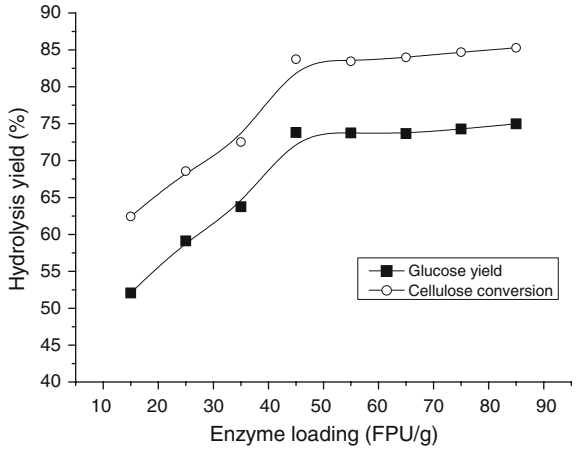

Fig. 3 Effect of the enzyme loading during the enzymatic hydrolysis of BSG cellulose for glucose obtainment

and especially cellobiose (Wen et al. 2004; Vlasenko et al. 1997). In the present work, the inhibition effect was probably due to the high glucose concentration in the hydrolysates ($\cong$59 g/L), which was approximately 8-fold higher than those of cellobiose.

Although the cellulose conversion has been incomplete, 59 g/L is a glucose concentration suitable for use in fermentative processes. Regarding the cellobiose concentration (6.7–7.9 g/L), it could be minimized by performing the hydrolysis with the cellulase complex supplemented with β-glucosidase, enzyme responsible for the cellobiose conversion into glucose (Cao and Tan 2002). Nevertheless, the high

cost due to addition of another enzyme would not justify the use of β-glucosidase to convert this low cellobiose concentration remaining in the hydrolysate. According to Chen et al. (2007), the enzyme cost significantly contributes to the total cost of biomass conversion process, and thus, the enzyme dosage must be minimized as much as possible. In addition, depending on the fermentative process, the cellobiose presence in the medium could not affect the microorganism performance. Moldes et al. (1999), for example, verified cellobiose consumption from pretreated wood hydrolysate during the lactic acid production by *Lactobacillus delbrueckii* NRRL B-445.

Another fact that should be pointed is regarding to the formation of by-products during enzymatic hydrolysis. Low by-products formation is an important advantage of enzymatic hydrolysis compared to acid hydrolysis (Martín et al. 2002). In the present work it was not detected the presence of any by-product, including sugars (xylose and arabinose) and compounds toxic for microorganisms (furfural, hydroxymethylfurfural, acetic acid and phenolics) in the enzymatic hydrolysate, which only contained glucose as main sugar and cellobiose in small amounts. Nevertheless, this cannot be only attributed to the high specificity of the enzymes used for hydrolysis, but was also a consequence of the two pretreatment steps that the raw material was submitted. The high hemicellulose and lignin removal from BSG during these stages, and the extensive material washing with water before enzymatic hydrolysis resulted in a cellulose pulp practically free of contaminants, and with low hemicellulose and lignin contents. Such substrate characteristics together with the high specificity of the enzymes led to a hydrolysate without by-products. The absence of toxic compounds, particularly, makes this hydrolysate a potential substrate for use in bioconversion processes.

Conclusions

Agitation speed, enzyme loading and substrate concentration are variables of great influence on enzymatic hydrolysis of BSG cellulose, with the intensity of them decreasing in the following order: enzyme loading > substrate concentration > agitation speed. Using the statistical methodology, it was possible to define equations that describe the variations in the glucose yield and in cellulose conversion as function of these three variables. Under the optimal hydrolysis conditions (100 rpm, 45 FPU/g substrate, 2% w/v substrate concentration) a high glucose yield, 93.1%, was achieved corresponding to a cellulose conversion of 99.4%. These values are high when compared to other lignocellulosic materials and it was not necessary the use of severe hydrolysis conditions so that they could be attained (some other cellulosic materials require a higher enzyme loading, for example, to provide results similar to these here attained). The easiness of glucose release from BSG makes this substrate a raw material with great potential to be used in bioconversion processes. Converting BSG into value-added products provides a potential alternative for treatment and disposal of such material.

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