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Comparison of Two Posthydrolysis Processes of Brewery's Spent Grain Autohydrolysis Liquor to Produce a Pentose-Containing Culture Medium†

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Abstract

A readily fermentable pentose-containing hydrolysate was obtained from Brewery's spent grain by a two-step process consisting of an autohydrolysis (converting the hemicelluloses into oligosaccharides) followed by an enzymatic or sulfuric acid–catalyzed posthydrolysis (converting the oligosaccharides into monosaccharides). Enzymatic hydrolyses were performed with several commercial enzymes with xylanolytic and cellulolytic activities. Acid-catalyzed hydrolyses were carried out at 121°C under various sulfuric acid concentrations and reaction times, and the effects of treatments were interpreted by means of a corrected combined severity factor (*CS**), which varied in the range of 0.80–2.01. Under the tested conditions, chemical hydrolysis allowed higher pentose yields than enzymatic hydrolysis. Optimized conditions (defined by *CS** = 1.10) allowed both complete monosaccharide recovery and low content of inhibitors. Liquors subjected to posthydrolysis under optimal conditions were easily fermented by *Debaryomyces hansenii* CCMI 941 in semiaerobic shake–flask experiments, leading to xylitol and arabitol as major fermentation products. The bioconversion process was improved by hydrolysate concentration and supplementation of fermentation media with casamino acids.

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Index Entries: Arabitol; *Debaryomyces hansenii*; hemicellulose hydrolysate; combined severity; xylitol.

Introduction

Brewery's spent grain (BSG) is the residue of malt and grain remaining in the mash kettle after the liquefaction and saccharification of starch to produce the wort. Since the manufacture of just 1 L of beer leads to about 0.04 kg of dry BSG, this material is largely available throughout the year. BSG is only used in low added-value applications such as feed, and therefore upgrading solutions are needed.

Even though the chemical composition of BSG may vary depending on the brewing conditions and ingredients used, its content of polysaccharides is remarkable (between 40 and 56% dry wt). Since hemicellulose constitutes the major fraction of BSG polysaccharides *(1–4)*, there arises the possibility of manufacturing hemicellulosic hydrolysates from BSG that could be utilized as fermentation medium, following the same principles already studied for the bioconversion of other agricultural or agroindustrial residues (such as corn cobs or sugarcane bagasse) into fuel (e.g., ethanol) or fine chemicals (e.g., polyols such as xylitol or arabitol) *(5–8)*. The remaining solid residue from BSG processing (made up of cellulose and lignin) could be upgraded by further processing as has been proposed for other raw materials *(7,9–11)*.

Several technologies for hemicellulose hydrolysis are available, including direct dilute-acid hydrolysis *(12)*, steam explosion *(13)*, and autohydrolysis *(2)*. The latter two are less severe than dilute-acid processes, and present several advantages, namely limited solubilization of lignin, low generation of degradation products, and low usage of chemicals, all of which are positive economic and environmental factors *(14)*. However, steam explosion and autohydrolysis lead to large amounts of oligomeric saccharides *(2)*, which have to be converted into monosaccharides (by acid or enzymatic catalysis) before fermentation. Studies exploring similar strategies for other raw materials have been reported *(15–17)*.

The main advantage of enzymatic posthydrolysis over the acidic process is the milder operation, which leads to reaction medium free of sugar- or lignin-degradation compounds that can limit microbial performance. Conversely, the acid process is faster, and the catalyst is cheap and allows high monosaccharide recovery. To make a correct choice, the performance of the raw material under consideration must be studied for each process.

The main factors affecting monosaccharide recovery in dilute-acid hydrolysis are catalyst concentration, reaction time, and temperature, whereas enzymatic hydrolysis is also dependent on additional factors such as substrate structure, and type and ratio of enzymatic activities present in the commercial enzyme preparations. The most important enzymes for xylan hydrolysis are endo-1,4-β-xylanases (which attack the main chain); β-xylosidases (which hydrolyze xylooligosaccharides to xylose); and accessory enzymes, such as acetyl xylanesterases, α-glucu-

ronidases, and α-arabinofuranosidases (which liberate substituents from the main chain). These enzymes act synergistically, since the xylanase activity is highly dependent on the presence of debranching enzymes and vice versa *(1,17–19)*.

The present work deals with a two-step procedure of autohydrolysis followed by posthydrolysis to obtain a fermentable, monosaccharide-rich liquor from the hemicellulose fraction of BSG. The autohydrolysis step was carried out under preoptimized conditions, and both enzymatic and chemical posthydrolysis were evaluated. Preliminary studies are also reported on the production of polyols from such liquor by *Debaryomyces hansenii*, a polyol-overproducing yeast *(20–25)*.

Materials and Methods

Feedstock and Pretreatment

BSG was obtained from Sociedade Central de Cervejas (Vialonga, Portugal). The raw material was mixed with water at an 8:1 (w/w) liquid-to-solid ratio and pretreated in an autoclave (Uniclave 88, AJC, Lisbon, Portugal) for 1 h at 100°C for residual starch removal. The solid was separated by filtration, washed and dried at 50°C to reach a moisture below 10% (w/w) *(2)*, homogenized to obtain a uniform lot, and stored in PA/PE vacuum-sealed bags.

Autohydrolysis

Starch-free BSG was subjected to reaction with water (autohydrolysis) in a 2-L stainless steel Parr reactor model 4532 (Moline, IL), to cause the hydrolytic degradation of hemicelluloses, operating under optimized conditions (liquid-to-solid ratio of 8:1 [w/w], standard heating temperature profile up to 190°C, isothermal reaction at 190°C for 2.5 min) *(2)*. After the reactor was cooled down, the oligosaccharide-containing liquor (OCL) was separated from the residual solid by filtration (Whatman no. 1 filter paper).

Posthydrolysis

Enzymatic Posthydrolysis

The pH of the OCL was adjusted to 5.5 using $Ca(OH)_2$, and the OCL was centrifuged at 6000*g* for 20 min (Beckman Coulter centrifuge; Fullerton, CA) and sterilized in an autoclave at 121°C for 15 min. Enzymatic hydrolyses were carried out in closed universal flasks at 35°C operating at 150 rpm in an orbital incubator (Unitron; Infors AG, Bottmingen, Switzerland) for 96 h. Commercial enzyme preparations were properly diluted in 0.05 *M* sodium citrate buffer (pH 5.5), and 1 mL of diluted enzyme solution was added to 25 mL of OCL. Seven commercial enzymatic preparations containing hemicellulolytic and cellulolytic activities were tested: Celluclast 1.5L®, Novozym 342®, Viscozyme L®, Pentopan 500BG® and Pulpzyme HC® from Novozymes, Denmark; and Multifect Xylanase® and

Multifect GC® from Genencor, Rochester, NY. The enzyme solutions were characterized by the following activities: endo-1,4-β-xylanase, β-xylosidase, acetylesterase, α-L-arabinofuranosidase, and filter paper activity (FPase, which describes the overall cellulolytic activity). The dilutions were calculated to provide 100 U of xylanase/g of total xylose in the reaction medium. Hydrolysis reactions were stopped by boiling for 5 min to inactivate enzymes and clarified by centrifugation prior to analysis. A blank OCL sample was assayed identically to enzyme-treated OCL using water instead of the enzyme solution. Control experiments were carried out for each enzyme, in which buffer replaced the OCL. All the hydrolysis trials were performed in duplicate.

Chemical Posthydrolysis

Defined volumes of 72% (w/w) H_2SO_4 were added to OCL to reach final concentrations of 1, 2, 3, or 4% (w/w) sulfuric acid in the reaction medium. Hydrolyses were carried out in an autoclave at 121°C for 15 or 60 min. Heating time from 100 to 121°C was 7 min. pH and weight of solutions were measured before and after autoclaving. No water losses were detected during autoclave treatments. All hydrolyses were done at least in duplicate.

Microorganism and Growth Conditions

Microorganism

D. hansenii CCMI 941 *(26)* was used in all experiments. The yeast was maintained on YM-xylose agar slants containing 20 g/L of xylose, 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 20 g/L of agar.

Preparation of Media

The hydrolysate obtained by acid posthydrolysis with 2% sulfuric acid (15 min) was centrifuged as described above. To adjust pH of the hydrolysate to 5.5, NaOH, CaO, or $Ca(OH)_2$ was added. When needed, precipitates formed were removed by centrifugation as described above. To obtain a fermentation medium with a higher monosaccharide content, a concentration step (1.8-fold) was carried out in an evaporation system comprising a Syncore orbital shaker equipped with four evaporation flasks, a vacuum pump VAC® v-500, and a vacuum controller B-721 (all from Büchi, Flawil, Switzerland). The operational conditions were as follows: lower plate temperature, 100°C; upper plate temperature, 70°C; pressure, 200 mbar; stirring, 175 rpm; volume per flask, 100 mL. Under these conditions, the concentrated hydrolysates were obtained in about 3 h.

When required, media were supplemented with casamino acids (Difco, Detroit, MI) to a final concentration of 5 g/L. Four different media were tested: hydrolysate (H), supplemented hydrolysate (SH), concentrated hydrolysate (CH), and concentrated and supplemented hydrolysate (CSH). To prevent additional medium decomposition, all media were filter sterilized using 0.22 μm Gelman membrane filters (Pall, Ann Arbor, MI).

Growth Conditions

Two 24-h-grown slants were used to seed 150 mL of nonsupplemented and nonconcentrated medium in a 1000-mL baffled Erlenmeyer flask. After 24 h, the cell biomass was centrifuged at 8500*g* under sterile conditions in a Sigma 3K15 centrifuge (Osterode am Harz, Germany), and a similar flask was inoculated with all the cell biomass. After 24 h, the cell biomass was centrifuged again and used to seed the final culture, in an initial cell dry weight concentration of about 2.8 g/L. All experiments were carried out aerobically in 500-mL Erlenmeyer flasks capped with cotton wool stoppers containing 200 mL of medium in an Infors Unitron orbital incubator set at 30°C and 150 rpm. All assays were done at least in duplicate.

Enzymatic Activities

All enzyme assays were carried out at 35°C in 0.05 *M* sodium citrate buffer (pH 5.5) similar to the conditions used for the enzymatic hydrolyses of OCL. Endo-1,4-β-xylanase (EC 3.2.1.8) and FPase activities were determined by measuring (after an incubation time of 30 min) the release of reducing sugars from the following substrates: 1% (w/v) oat spelts xylan (Sigma, Steinheim, Germany) and Whatman no. 1 filter paper (about 50 mg), respectively. Reducing sugars released were assayed by the dinitrosalicylic acid method *(27)*.

β-D-Xylosidase (EC 3.2.1.37), acetylesterase (EC 3.1.1.6), and α-L-arabinofuranosidase (EC 3.2.1.55) activities were determined using the synthetic substrates 1 m*M* *p*-nitrophenyl-β-D-xylopyranoside, 5 m*M* *p*-nitrophenyl-acetate, and 5 m*M* *p*-nitrophenyl-α-L-arabinofuranoside (all from Sigma). Release of *p*-nitrophenol was monitored spectrophotometrically at 410 nm using a Jasco V-530 Spectrophotometer.

One unit of enzymatic activity was expressed as 1 μmol of xylose, glucose, or *p*-nitrophenol released per minute under the specified conditions.

Analytical Methods

D-Xylose; D-glucose; L-arabinose; formic, acetic, and levulinic acids; ethanol; hydroxymethylfurfural (HMF), and furfural were quantified by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column from Bio-Rad (Hercules, CA). The HPLC system was a Waters LC module 1 plus (Millford, MA) equipped with both a refractive index and an ultraviolet detector set at 280 nm (used to detect HMF and furfural). The mobile phase was 5 m*M* H_2SO_4, the column temperature was 50°C, and the flow rate was 0.4 mL/min. The system was equipped with a Micro-Guard Cation-H Refill Cartridge from Bio-Rad prior to the HPX-87H column. In the fermentation experiments, owing to the partial overlap of arabinose, xylitol, and arabitol, samples were also analysed by HPLC using a Waters Sugar Pak 1 column. It was used with a Merck Hitachi HPLC system (Tokyo, Japan) equipped with a refractive index detector (L-7490).

The mobile phase was 50 mg/L of calcium EDTA, the column temperature was 90°C, and the flow rate was 0.5 mL/min. Once this method no longer allowed us to distinguish between D- and L-arabitol, the latter was used as arabitol standard. All samples were filtered with 0.45-µm Gelman membrane filters prior to analysis.

Total phenolic compounds content was assayed spectrophotometrically using a modified Prussian blue method as described by Graham *(28)* employing an LKB Biochrom Ultrospec II spectrophotometer (Cambridge, England). Tannic acid was used as calibration standard.

Cellular dry weight concentration was quantified gravimetrically by filtering of 5 mL of culture broth through 0.45-µm Gelman membrane filters, washing with an equal volume of water, and drying overnight at 100°C to constant weight.

Calculations

Monosaccharide, aliphatic acids, furan derivatives, and phenolic compound recoveries after posthydrolysis were calculated as the ratio between the concentration determined in the reaction media and the concentration that resulted from the quantitative acid hydrolysis *(29)* of oligosaccharides into monosaccharides and other compounds. In enzymatic treatments, the concentrations obtained were corrected by subtracting the corresponding concentration in the respective control assays, since the commercial enzymes contain mono-, di-, and oligosaccharides. The dilution factor introduced by adding the dilute enzyme preparation or the different volumes of sulfuric acid in posthydrolysis were also accounted for.

In the same way as reported in the literature *(30)*, the severity of operational conditions in acid post hydrolysis was comparatively assessed by means of a combined severity parameter *CS** (*see* below).

The volumetric substrate consumption rates, Q_s (g/[L·h]), were based on grams of substrate consumed per liter of culture medium/h at designated times. The product volumetric production rate (productivity), Q_p (g/[L·h]), was based on grams of product produced per liter of culture medium/h at designated times. The product yield, $Y_{p/s}$ (g/g), was calculated as the amount of product formed per gram of designated consumed substrates.

Results and Discussion

Autohydrolysis enables the selective hydrolysis of hemicelluloses to a mixture mainly consisting of oligosaccharides and monosaccharides. The monosaccharide content can be increased under harsher reactor conditions, but then monosaccharides can undergo decomposition reactions, thereby increasing the content of potential fermentation inhibitors in hydrolysates.

Under the BSG autohydrolysis conditions used herein, most of the hemicelluloses are converted mainly into oligosaccharides. To obtain a monosaccharide-rich hydrolysate, the OCLs have to be further hydrolyzed.

Two different processes (enzymatic posthydrolysis and acid posthydrolysis) were assayed for this purpose.

Enzymatic Posthydrolysis

Unlike starch or cellulose, BSG hemicellulose has a complex structure, which is still present, in part, on its autohydrolysis products. Oligosaccharides from BSG hydrolysis consist mainly of branched arabino-xylo-glucurono oligosaccharides that are not highly acetylated when compared to other xylans, such as from *Eucalyptus* wood *(31)*. The action of several enzymatic activities including endo-1,4-β-xylanase; β-xylosidase; and accessory activities such as acetyl xylanesterase, α-glucuronidase, and α-arabinofuranosidase is therefore required for the complete hydrolysis of OCL to monosaccharides.

Table 1 shows the main activities present in the various reaction mixtures using Celluclast 1.5L, Novozym 342, Viscozyme L, Pentopan 500BG, Pulpzyme HC, Multifect Xylanase, and Multifect GC. Although most endoxylanases present in the commercial preparations exhibit optimal activity near 50°C, hydrolysis assays were carried out at a lower temperature (35°C) to increase enzyme stability and to reduce the possibility of inactivation of other enzymatic activities having unknown stability profiles.

The monosaccharides and acetic acid recoveries obtained on enzymatic posthydrolysis are also provided in Table 1. The concentrations of formic and levulinic acids, HMF, and furfural concentrations were also determined, but in all cases, they remained unchanged throughout the enzymatic process. The same trend was observed for the total phenolic content (data not shown).

The lowest recovery for monosaccharides and acetic acid was obtained with Pulpzyme HC. This can be explained by the fact that this preparation only exhibits endo-1,4-β-xylanase activity, lacking the debranching enzymatic activities. The best total sugar recovery was obtained using either Celluclast 1.5L or Viscozyme L, enabling about 75% arabinose recovery, 63% xylose recovery, and almost total glucose recovery, with no additional production of microbial inhibitors, except acetic acid. Because BSG oligosaccharides are not highly acetylated, a residual acetyl xylanesterase activity may be sufficient to completely remove the linked acetyl groups.

The incomplete degradation of the main xylan backbone might be partially explained by its complex structure having several steric hindrances. On the other hand, the ratios of the assayed enzymes present in the commercial preparations tested might not be adapted to the structure of the BSG-derived oligosaccharides, suggesting that improved yields could be obtained by using mixtures of these preparations and, additionally, by applying sequential enzyme treatments *(32)*.

Besides these factors, the synthetic nitrophenyl derivatives used for estimating β-xylosidase, acetyl xylanesterase, and α-arabinofuranosidase activities may not provide suitable information to be applied to a real, complex substrate and/or additional enzymes (such as α-glucuronidases)

Table 1
Enzyme Activity Levels Present in Reaction Mixture for Different Commercial Enzyme Preparations Used and Respective Monosaccharides and Acetic Acid Recoveries for Enzymatic Posthydrolysis of BSG OCL[a]

	Enzyme activities (U/g xylose)					Recovery (%)			
Enzyme formulations	β-xylanase	β-xylosidase	Acetyl esterase	α-arabino-furanosidase	FPase	Glucose	Xylose	Arabinose	Acetic Acid
Celluclast 1.5L	100	0.53	ND	3.41	3.84	94	63	77	115
Novozym 342	100	0.04	0.09	0.09	0.25	52	36	79	92
Viscozyme L	100	0.08	0.52	37.91	2.01	119	63	74	105
Pentopan 500BG	100	0.69	ND	1.45	0.63	44	32	77	96
Pulpzyme HC	100	ND	ND	ND	vest.	9	12	52	68
Multifect Xylanase	100	vest.	0.01	0.01	0.03	9	28	60	86
Multifect GC	100	0.12	0.02	0.13	1.95	13	46	69	90

[a]The data are relative concentrations compared to the quantitative acid hydrolysis *(29)*. ND, not detected; vest., <0.01 U/g of xylose.

could play a role in saccharification. Finally, possible inhibitory effects on enzymatic activities caused by the phenolic compounds present in OCL could limit the extent of oligosaccharide saccharification *(18)*.

Acid Posthydrolysis

Acid posthydrolysis was studied as an alternative to enzymatic posthydrolysis, since limited monosaccharide recoveries were achieved with that technology. When compared to the enzymatic hydrolysis process, significant monosaccharide degradation reactions may occur during acid posthydrolysis. Examples of such reactions are the degradation of pentoses to furfural, hexoses to HMF, and both these furans to aliphatic acids such as formic and levulinic acids. Therefore, high monosaccharide recovery requires balanced operational conditions. In our case, the severities of treatments were modified by varying the concentrations of sulfuric acid (in the range of 1–4 % [w/w]) and reaction times of 15 or 60 min.

To provide a comparative assessment on the effects caused by the severity of operational conditions in acid-catalyzed media, the joint effects of temperature, reaction time and catalyst have been interpreted in terms of the parameter *CS (30)*. For isothermal conditions,

$$CS = \log\left\langle t \exp\left[(T - 100)/\omega\right]\right\rangle - \mathrm{pH} \quad (1)$$

in which t is the isothermal reaction time (min), T is the temperature (°C), and ω is a parameter dependent on the activation energy of the reaction that usually is assumed to be 14.75.

The parameter *CS* is an extension of the severity concept *(33)* that has been mainly used for the hydrolysis of lignocellulosic biomass with chemically defined catalysts. However, the application of *CS* to OCL hydrolysis might not be straightforward owing to the necessity of setting a suitable pH for the reaction. It can be noted that pH cannot be calculated from the molar H_2SO_4 concentration considering that 2 mol of H_3O^+/mol of sulfuric acid is produced, as generally assumed *(34,35)*. This may be justified by the facts that OCL has a complex polyelectrolite nature, that compounds with buffering activity exist in the reaction media and that side reactions involving pH alteration (e.g., hydrolysis of acetyl groups) occur throughout the posthydrolysis process. Considering this, and on the basis of the pK_a for the second dissociation of sulfuric acid for the temperatures and catalyst concentrations used in the present work, a simplified combined severity parameter (*CS**) was defined as:

$$CS^* = \log\left\langle t \exp\left[(T - 100)/\omega\right]\right\rangle - \log\left[\mathrm{H_2SO_4}\right] \quad (2)$$

in which $[H_2SO_4]$ is the molar concentration of sulfuric acid. It can be noted that *CS* and *CS** take the same value if 1 mol of (H_2SO_4) results in the net release of 1 mol of H_3O^+. This hypothesis was supported by the fair agreement found between the pH of post-hydrolyzed media measured experimentally at room temperature and the pH calculated assuming a net release

Table 2
Operational Conditions, *CS**, and Relative Composition of Posthydrolysis Liquors (Measured by Monosaccharides and Furan Derivatives) Expressed as Percentages of Concentrations Obtained on Quantitative Acid Hydrolysis (*CS** = 2.01)[a]

Operational conditions			Relative composition[b] (%)				
H_2SO_4 (% [w/w])	Time (min)	*CS**	Glucose	Xylose	Arabinose	HMF	Furfural
1	15	0.80	28	70	101	42	38
2	15	1.10	81	105	102	61	63
3	15	1.28	90	107	105	60	64
4	15	1.41	96	105	102	68	66
4	60	2.01	100	100	100	100	100

[a]All posthydrolysis assays were carried out at 121°C.
[b]Aliphatic acids content and total phenolic compounds did not change significantly with severity and were omitted from the table.

of 1 mol of H_3O^+/mol of sulfuric acid. Additional calculations showed that the values of *CS** corresponding to the heating stages were negligible in comparison with the corresponding results determined for the isothermal reaction stage.

Table 2 shows the operational conditions, the respective *CS**, and the chemical composition of BSG posthydrolysis liquors for the different posthydrolysis conditions tested. Compositions of posthydrolyzed liquors were expressed as percentages regarding to the concentrations determined by quantitative acid hydrolysis *(29)*.

Under the mildest operational conditions tested, arabinose was totally solubilized and not significantly degraded to furfural (a fact that may explain the 101% recovery with respect to the quantitative acid hydrolysis conditions), but both xylose and glucose recoveries were rather low. Sugar recovery increased with increasing severity, reaching a maximum for pentoses at a *CS** of 1.28. Further increases in severity led to pentose degradation, as indicated by higher furfural concentrations, with a negative impact in pentose recovery. Conversely, glucose recoveries always increased with the increase in severity, indicating that the gluco-oligosaccharides were more difficult to hydrolyze than the hemicellulosic oligosaccharides. The levels of aliphatic acids and total phenolic compounds were also measured, but only slight concentration changes were detected (data not shown). Aliphatic acids exhibited average concentrations of approx 1.3 g of acetic acid/L, 0.5 g of formic acid/L, and <0.2 g of levulinic acid/L, with minor increases with respect to their concentrations in autohydrolysis of OCL *(2)*. The concentrations of total phenolic compounds obtained after acid posthydrolysis were also similar to the results determined in autohydrolysis of OCL, indicating that these compounds do not react significantly during acid posthydrolysis.

Although the higher recovery of pentoses was obtained at *CS** 1.28, if the dilution effect (owing to the addition of acid) is accounted for, the recovery of pentoses obtained at *CS** 1.10 becomes similar to that obtained at *CS** 1.28, whereas only slightly lower glucose recovery was achieved. Since aliphatic acid and furan derivative recoveries were also similar, the least severe condition should be considered the most adequate condition for acid posthydrolysis, once it decreases acid and later also alkali requirements, both with a positive impact on process economics.

Similar conditions have been described for posthydrolysis of steam-exploded Douglas fir wood chips performed at 120°C, but longer hydrolysis time was required *(15)*. Similar to our results, increasing catalyst also increased monosaccharide recovery and higher participation of degradation reactions. For corn cobs *(36)*, a material similar to BSG, the posthydrolysis of OCL autohydrolysis was carried out at *CS** 1.66 (calculated from the reported operational conditions: 125°C, 0.5% H_2SO_4, 165 min), a more severe condition than the optimal conditions determined in the present work for BSG.

The total monosaccharide composition of OCL acid posthydrolysate is approx 26 g/L of monosaccharides, with a ratio of 2:5:3 for glucose: xylose:arabinose. Compared to other hydrolysates, it has higher arabinose but lower xylose contents than rice straw *(37)*, sugarcane bagasse *(38)*, *Eucalyptus* wood *(16)*, and corncobs *(36)*, where the latter two hydrolysates were also obtained in a similar two-step procedure. Nevertheless, the total monosaccharide content is in the same range, e.g., as rice straw, *Eucalyptus* wood, or corncobs.

A significant comparative advantage of this BSG hydrolysate lies with the low level of microbial inhibitors; it is among the lowest reported in the literature *(16,37)*. In fact, the more relevant inhibitors usually considered for lignocellulosic-based hydrolysates, acetic acid and furfural, are present at 1.2 and 0.6 g/L, respectively, which is lower than the levels of microbial inhibition presented in the literature, at 4 and 1 g/L respectively *(39)*.

Polyol Production by D. hansenii

Polyols, namely arabitol and xylitol, have potential chemical, pharmaceutical, and food applications. The latter polyol is currently produced by chemical means, in spite of xylitol bioproduction receiving increased interest *(40)*. Previously it was shown *(22)* that *D. hansenii* CCMI 941, under oxygen limitation conditions, coproduces both of arabitol and xylitol in chemically defined medium. In the present work, we evaluated the polyol production by *D. hansenii* grown on the BSG acid posthydrolysate.

Although other researchers have reported the use of detoxification steps for other hydrolysates to be utilized by yeast *(5,7,38,41)*, considering the low level of inhibitors present in the BSG OCL acid posthydrolysate, no special detoxification treatment was carried out, except adjustment of the hydrolysate pH to 5.5. For pH adjustment of the hydrolysate, several alkalis were tested, namely NaOH, CaO and $Ca(OH)_2$. CaO, induced high foam

formation during pH adjustment, whereas NaOH-treated hydrolysates turn to a gel-like consistency during the evaporation step used to obtain the concentrated hydrolysates. Therefore, $Ca(OH)_2$ was chosen for the pH adjustment because it did not present any significant operational problems, and it limited the loss of monosaccharides (<6%), while providing complete removal of levulinic acid and a partial removal of HMF (9%), furfural (15%), and phenolic compounds (10%).

Figure 1 shows the kinetics of polyol production by *D. hansenii* grown in both hydrolysate medium (Fig. 1A) and concentrated hydrolysate medium (Fig. 1B). During the hydrolysate concentration, a minor loss of monosaccharide occurred and furfural was evaporated. All other components were concentrated, although some components such as acetic acid were partially lost during evaporation. Significant foam formation was observed during evaporation and vacuum filtration.

During fermentation glucose was the preferred monosaccharide, being depleted from the culture medium in about 48-h. Concomitantly, HMF, furfural, and formic acids were also metabolized (data not shown). Acetic acid was slowly consumed compared with formic acid (data not shown). Xylose was consumed after a short lag phase (Fig. 1A) albeit at a slower rate compared to glucose assimilation. Arabinose was only slightly consumed. Arabitol was the major polyol formed for both hydrolysate and concentrated hydrolysate media. Xylitol was also produced, but at a far lesser extent. Glycerol, another important metabolite usually accumulated in *D. hansenii*, was not detected. Ethanol was produced mostly at the expense of glucose consumption, and after glucose depletion no further ethanol accumulation occurred from pentose catabolism. Conversely, some ethanol was presumably oxidized to biomass and CO_2 concomitantly with the pentose metabolism.

Previous reports in the literature suggest that media supplementation with complex nutrient sources can increase both xylitol yield and productivities *(41)*. Therefore, a second set of experiments were carried out by adding casamino acids to both hydrolysate (Fig. 2A) and concentrated media (Fig. 2B). In both conditions, casamino acids induced changes in the yeast metabolism in relation to nonsupplemented media. Besides glucose, both xylose and arabinose were consumed more quickly and a simultaneous consumption of glucose-xylose and xylose-arabinose was observed, as already described for this yeast when grown in chemically defined media *(22,42)*. Table 3 shows the yields and volumetric rates for the metabolic products from *D. hansenii* grown under different hydrolysate media compositions. The xylose consumption rate was higher for supplemented media being for casamino acids–supplemented hydrolysate and concentrated and supplemented hydrolysate media, 0.19 and 0.30 g/(L·h), respectively. HMF, formic acid, and acetic acid were consumed slightly faster in these experiments, and again formic acid was depleted before acetic acid (data not shown).

Ethanol volumetric production rate did not differ significantly between nonsupplemented and supplemented media, but ethanol was no longer

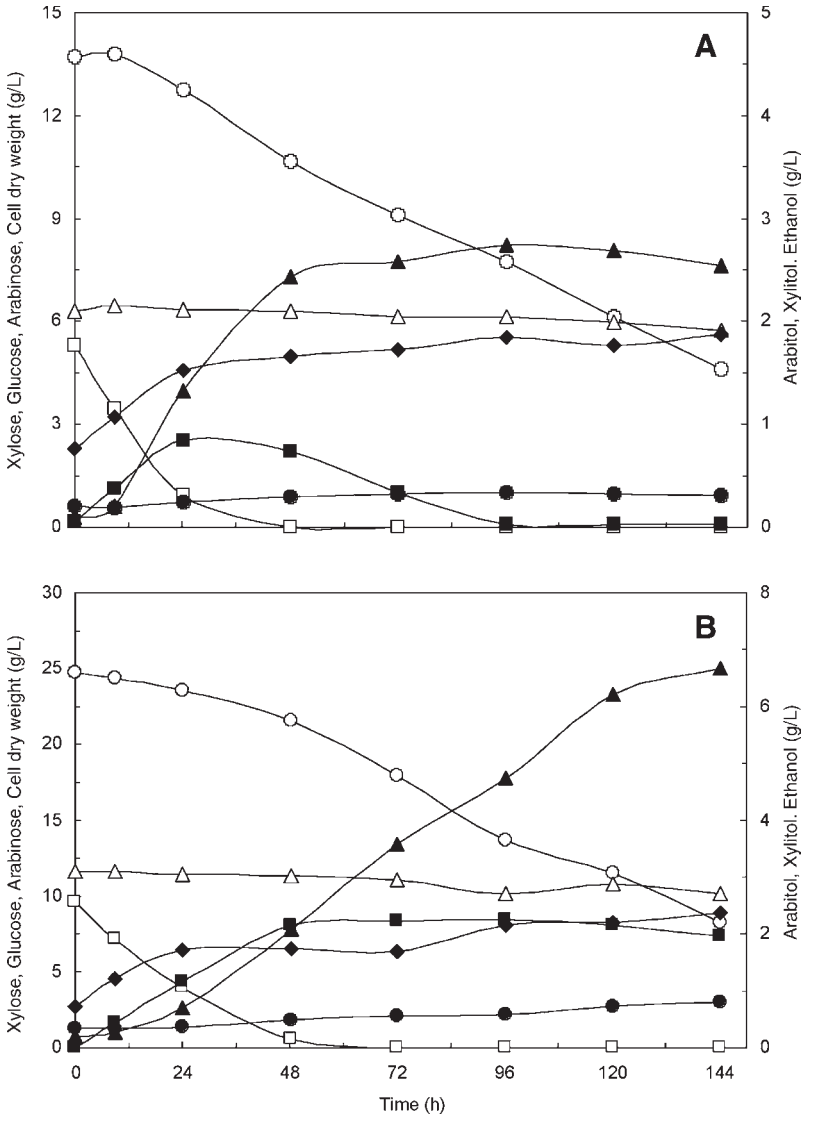

Fig. 1. Time course of substrates, cell dry weight, and metabolic products in fermentation of BSG OCL posthydrolysate (H medium) **(A)** and concentrated posthydrolysate (CH medium) **(B)** by *D. hansenii* CCMI 941. (□) Glucose, (○) xylose, (△) arabinose, (◆) cell dry weight, (■) ethanol, (●) xylitol, and (▲) arabitol.

exclusively derived from glucose metabolism for supplemented medium. In the latter medium, pentose metabolism becomes the greater contributor to ethanol production. Although ethanol production in this strain has been mainly correlated with glucose metabolism, ethanol from pentoses has also already been reported *(22)*. Arabitol was accumulated almost throughout the fermentation, but in both cases, when sugars were close to depletion it was also used as a carbon source.

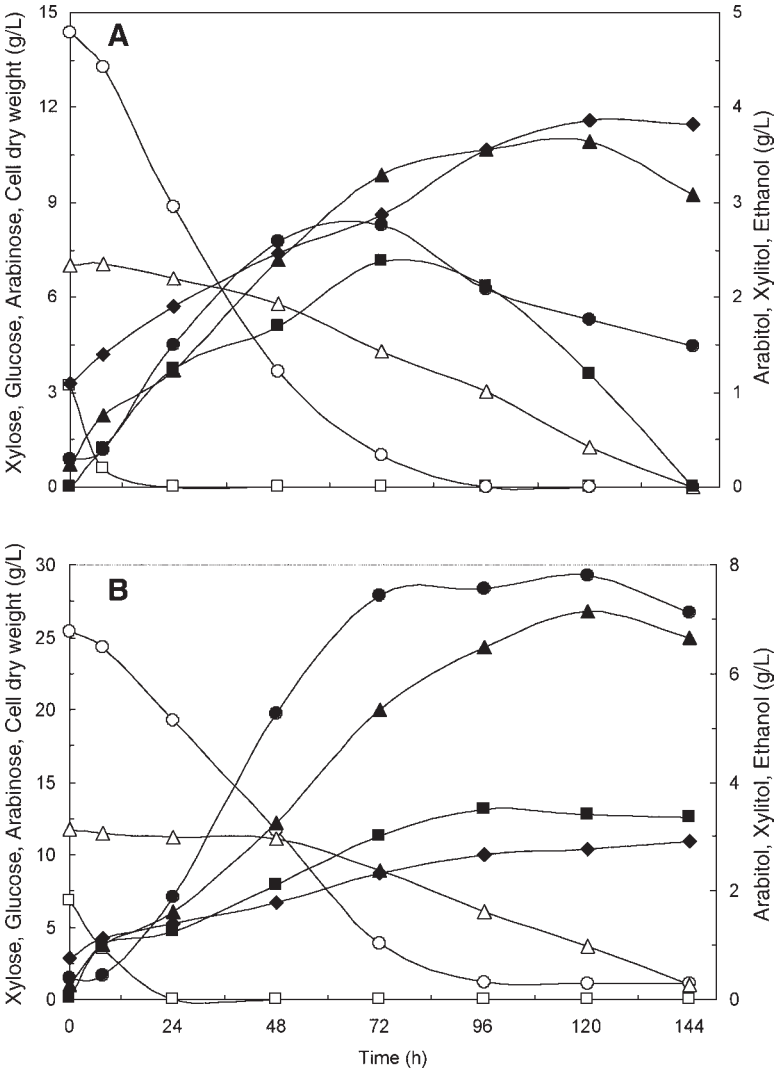

Fig. 2. Time course of substrate, cell dry weight, and fermentation products in fermentation of casamino acids supplemented with BSG OCL posthydrolysate (SH medium) **(A)** and casamino acids supplemented concentrated posthydrolysate (CSH medium) **(B)** by *D. hansenii* CCMI 941. (□) Glucose, (○) xylose, (△) arabinose, (◆) cell dry weight, (■) ethanol, (●) xylitol, and (▲) arabitol.

The major change in yeast metabolism induced by casamino acids supplementation was the marked increase in xylitol production, being the major metabolic product by *D. hansenii* grown in supplemented concentrated medium (Fig. 2B). Both arabitol and xylitol production in yeast are described to be augmented under stress conditions. Arabitol is usually found as a product of arabinose metabolism in oxygen-limited conditions *(8,22)*, but arabitol production is not restricted to arabinose metabolism,

Table 3
Maximal Volumetric Production Rates and Yields for Cell Biomass, Ethanol, Arabitol, and Xylitol Obtained with *D. hansenii* CCMI 941 Yeast for Different Tested Conditions

	Ethanol			Arabitol			Xylitol		
Exp[a]	Time (h)	Q_p (g/[L·h])	$Y_{p/s}$ (g/g)	Time (h)	Q_p (g/[L·h])	$Y_{p/s}$ (g/g)	Time (h)	Q_p (g/[L·h])	$Y_{p/s}$ (g/g)
H	24	0.03	0.18[b]	48	0.05	0.77[c]	—	0.00	0.00
CH	48	0.04	0.24[b]	120	0.05	0.45[c]	—	0.00	0.00
SH	72	0.03	0.12	72	0.04	0.19[d]	72	0.03	0.15[d]
CSH	72	0.04	0.10	72	0.07	0.21[d]	72	0.10	0.29[d]

[a] H, hydrolysate; CH, concentrated hydrolysate; SH, casamino acids–supplemented hydrolysate; CSH, concentrated and supplemented hydrolysate.
[b] Based on consumed glucose.
[c] Based on consumed xylose.
[d] Based on consumed pentoses.

since it has also been described for *Saccharomyces cerevisiae* grown in anoxic conditions in a glucose/xylulose mixture *(43)* and for *Pichia stipitis* grown in xylose with a severe respiratory inhibition *(44)*. Furthermore, its production as a compatible solute in the yeasts *Zygosaccharomyces rouxii* and *D. hansenii* is also well documented, where arabitol accumulation is part of the yeast response to osmotic stress, regardless of the carbon source being utilized *(23,45,46)*.

Thus, the relatively constant arabitol production in all tested conditions can probably be explained by a combination of factors, such as high oxygen limitation and possible osmotic effects (e.g. induced by sulfate ions not fully precipitated with calcium) that may act synergistically. Arabitol yields and productivities found in the present work are similar to reported values in the literature *(8,22)*.

On the other hand, higher xylitol production is usually correlated to, among other factors, oxygen limitation, high xylose concentrations and high xylose uptake rates *(41)*. Casamino acids induce an increase in xylose uptake rate, therefore increasing xylitol production that is further enhanced by the hydrolysate concentration. Although the maximal obtained yields and volumetric production rates for xylitol are in the range usually described for this yeast *(42)*, they are below the reported values for optimized conditions *(25)*, which for xylitol production usually requires higher xylose concentrations and oxygen availability. Therefore, this system can still be improved in order to further increase both polyol yields and productivities.

Conclusions

Enzymatic posthydrolysis enabled monosaccharide recoveries in the range usually attained for other feedstocks to be obtain *(17,18)*. The higher recoveries were obtained with Celluclast 1.5L and Viscozyme L, with arabinose recoveries close to 75%, xylose recoveries of 63%, and close to total glucose recovery. Furthermore, no additional production of microbial inhibitors occurs during the enzymatic step, with the exception of acetic acid. However, the long incubation time and catalyst costs may constitute important constraints for the near-term implementation of an enzyme-based conversion process to the conversion of OCL to monosaccharides.

Since dilute-acid posthydrolysis with 2% (w/w) H_2SO_4 at 121°C for 15 min enables hydrolysis of the oligosaccharides with a good recovery of pentoses and only a slight increase in microbial inhibitors, no attempt was made to optimize the enzymatic process (namely, in terms of enzyme dosage, incubation time or by applying mixtures of enzymes). The acid posthydrolysate was therefore used for *D. hansenii* cell biomass and polyol production without any detoxification treatment, except pH adjustment to 5.5. Hydrolysate concentration led to higher polyol productivities, and supplementation with casamino acids favored xylitol production, pointing out a possible direction for further studies in polyol production optimization. *D. hansenii* is a particularly interesting yeast for that purpose since the ratio between both polyols can be modulated by simple manipulation of culture conditions.

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