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Supplementation requirements of brewery's spent grain hydrolysate for biomass and xylitol production by *Debaryomyces hansenii* CCMI 941

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Abstract The effect of nutrient supplementation of brewery's spent grain (BSG) hydrolysates was evaluated with respect to biomass and xylitol production by *Debaryomyces hansenii*. For optimal biomass production, supplementation of full-strength BSG hydrolysates required only phosphate (0.5 g l^{-1} KH_2PO_4), leading to a biomass yield and productivity of 0.60 g g^{-1} monosaccharides and 0.55 g l^{-1} h^{-1}, respectively. Under the conditions studied, no metabolic products other than CO_2 and biomass were identified. For xylitol production, fourfold and sixfold concentrated hydrolysate-based media were used to assess the supplementation effects. The type of nutrient supplementation modulated the ratio of total polyols/total extracellular metabolites as well as the xylitol/arabitol ratio. While the former varied from 0.8 to 1, the xylitol/arabitol ratio reached a maximum value of 2.6 for yeast extract (YE)-supplemented hydrolysates. The increase in xylitol productivity and yield was related to the increase of the percentage of consumed xylose induced by supplementation. The best xylitol yield and productivity were found for YE supplementation corresponding to 0.55 g g^{-1} and 0.36 g l^{-1} h^{-1}, respectively. In sixfold concentrated hydrolysates, providing that the hydrolysate was supplemented, the levels of xylitol produced were similar or higher than those for arabitol. Xylitol yield exhibited a further increase in the sixfold hydrolysate supplemented with trace elements, vitamins and minerals to 0.65 g g^{-1}, albeit the xylitol productivity was somewhat lower. The effect of using activated charcoal detoxification in non-supplemented versus supplemented sixfold hydrolysates was also studied. Detoxification did not improve polyols formation, suggesting that the hemicellulose-derived inhibitor levels present in concentrated BSG hydrolysates are well tolerated by *D. hansenii*.

Keywords *Debaryomyces hansenii* · Xylitol · Polyols · Brewery's spent grain · Hemicellulosic hydrolysate · Nutrient supplementation

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Introduction

The use of hemicellulosic hydrolysates in bioprocesses such as xylitol production is one possible approach to upgrading xylan-rich lignocellulosic residues. Xylitol production by yeast is favoured by high initial xylose concentrations, oxygen limitation, high cell inoculum level and appropriate medium supplementation [27, 29, 41]. Moreover, the xylitol bioprocess should preferably be carried out in a two stage process: a first step of biomass production carried out on a full-strength hydrolysate-based medium with oxygen excess, and a subsequent step of xylitol production based on concentrated (high xylose level) hydrolysate and oxygen-limiting conditions for producing xylitol [28, 33, 38].

Among the several agro-industrial residues that can be used to produce xylose-rich media, brewery's spent grain (BSG) hydrolysates have been reported to be easily and readily utilised by the yeast *Debaryomyces hansenii* [4, 10]. BSG hydrolysate also has the advantage over other lignocellulosic hydrolysates because there is no absolute need for a preliminary detoxification step, to produce either yeast biomass (in full-strength media) or xylitol (in concentrated media) [3]. Nevertheless, supplementation is of paramount importance to increase fermentation performance (biomass and xylitol yields and productivities). Therefore, it is important to identify both the type and the minimal supplement requirements

to minimise the costs involved in media formulation for both the first and second steps of the xylitol bioprocess. Furthermore, in some cases, high concentrations of nutrients may favour cell growth to the detriment of xylitol production [40], which highlights the need for an accurate medium formulation. This knowledge is also important to further the progress towards xylitol industrial application by minimizing nutrient addition and simplifying the fermentation process design.

In the optimisation of hemicellulosic hydrolysates-based media for biomass production, vitamins and trace element supplementation may not be required, since these compounds might already be present in sufficient amounts [30]. Conversely, nitrogen and/or phosphate are generally required for supplementing hydrolysates regardless of the yeast species [20, 24, 30]. For the xylitol production step, the type and concentration of the nitrogen source are crucial factors, but their influence strongly depends upon the yeast species and the composition of the hydrolysate. For example, aspen wood hydrolysate supplemented with yeast extract (YE) has been described to increase xylitol production by *Candida parapsilosis* [32]. A similar pattern was shown for *C. guillermondii* grown in sugar cane bagasse hydrolysate upon the addition of ammonium sulphate and rice bran extract [5]. The use of corn steep liquor (CSL), which is an inexpensive source of nitrogen, vitamins and other nutritional requirements, has also been reported to increase xylitol production when used as the sole supplement in *Eucalyptus* wood hydrolysates [8]. A positive effect of casamino acids (CAS) on xylitol production has been described for several yeasts, both for a chemically defined medium containing xylose as the carbon source [2, 9, 41] and also for hydrolysate-based media [10]. Conversely *C. guillermondii* was reported to decrease xylitol production after the addition of nitrogen sources to rice straw hydrolysate [39].

Previous work dealing with xylitol production by *D. hansenii* in hemicellulosic hydrolysates (e.g., *Eucalyptus* wood [8], corn cobs [34] and BSG [10]) suggest that hydrolysate supplementation is crucial to achieve high xylitol productivities and yields.

In this work, the supplementation of BSG hydrolysates for attaining optimal biomass levels was first optimised for each nutrient group used in media preparation: trace elements, vitamins as well as nitrogen, phosphate and magnesium sources. The formulated medium was further validated for its use as an industrial growth medium. The effect of several complex nutrients, both on xylitol and on total polyols production, in fourfold and sixfold concentrated BSG hydrolysates was also evaluated.

Materials and methods

Preparation of hemicellulosic hydrolysate

Brewery's spent grain hemicellulosic hydrolysate was obtained through a sequential process of autohydrolysis followed by a post-hydrolysis with dilute sulphuric acid as previously described [10]. The pH of the liquors obtained after post-hydrolysis was adjusted to pH 5.5 by the addition of solid $Ca(OH)_2$. After 1 h the precipitate was removed by centrifugation at 7,500*g* for 25 min (Beckman Coulter, Fullerton, USA). The pH-adjusted hydrolysate was used for growth experiments, and after concentration, for xylitol production experiments either with or without previous detoxification.

Detoxification

The pH 5.5 hydrolysate was treated with granular activated charcoal as described elsewhere [3]. The detoxified hydrolysate was recovered by filtration (filter paper Whatman no. 1).

Concentration

Non-detoxified and detoxified hydrolysates were concentrated by evaporation in order to reduce them to about 75 or 84% of the initial weight (fourfold and sixfold concentrations, respectively). Evaporation was performed using the evaporation system and operational conditions previously described [10] in order to have a liquid temperature of 60°C. When needed, the pH of the concentrated hydrolysates was adjusted to 5.5 with $Ca(OH)_2$ or H_2SO_4.

Microorganism

Debaryomyces hansenii (CCMI 941) was maintained at 4°C on YM-xylose agar slants containing (g l^{-1}): D-xylose, 20; YE, 3; malt extract (ME), 3; peptone (PEPT), 5; and agar, 20. Before use, fresh yeast cells were obtained after incubation at 30°C for 24 h.

Cultivation conditions

Growth experiments

For inocula preparation, *D. hansenii* fresh yeast cells from YM-xylose slants were used to inoculate 1-l baffled Erlenmeyer flasks containing 80 ml of the non-supplemented hydrolysate. The cultures were incubated in an Infors® Unitron orbital incubator (Bottmingen, Switzerland) at 150 rpm and 30°C for 18 h.

To study the effect of supplementation on yeast growth, the following supplements were added to the hydrolysate as follows: (a) trace elements, vitamins and/or minerals (nitrogen, phosphate and magnesium sources), at the final concentrations described previously [3, 25]; (b) nitrogen (9 g l^{-1} $(NH_4)_2SO_4$) or phosphate (2.5 g l^{-1} KH_2PO_4) or magnesium (0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$); and (c) up to 1.25 g l^{-1} KH_2PO_4. The minerals

and trace element stock solutions were autoclaved separately. The vitamin stock solution was sterilised using 0.22 μm Gelman membrane filters (Ann Arbor, MI, USA). The required volumes were added aseptically to filter-sterilised hydrolysates. In non-supplemented hydrolysates (negative controls), the volume of supplements was replaced by sterile water.

The inoculum culture (10 ml) was used to seed 1-l baffled Erlenmeyer flasks containing 80 ml of the non-supplemented or supplemented hydrolysate. All cultures were incubated for 24 h under the same conditions as the inoculum. At pre-determined fermentation times, samples were withdrawn for HPLC analysis and cell growth measurements. Each cultivation was repeated at least twice and the mean values are reported.

To test the ability of the optimised medium to sustain *D. hansenii* growth, the cells obtained after a cultivation run in phosphate-supplemented hydrolysate (0.5 g l^{-1} KH_2PO_4) were transferred consecutively and used to inoculate another medium with the same composition. The volumes of cell suspension added to the media were adequate to provide an initial biomass concentration around 1.5 g l^{-1}. Cultivation was performed under the same conditions as described for the growth experiments.

Xylitol production experiments

Fresh yeast cells from YM-xylose slants were used to pre-inoculate 1-l baffled Erlenmeyer flasks containing 80 ml of non-supplemented hydrolysate. Pre-inocula cultures were grown in an Infors® Unitron orbital incubator (Bottmingen, Switzerland) at 150 rpm and 30°C for 18 h. This pre-inoculum was further used to seed 1-l baffled Erlenmeyer flasks containing 80 ml of hydrolysate supplemented with 0.5 g l^{-1} KH_2PO_4. The culture was incubated under the same conditions as described before. After 18 h, the cells were harvested by sterile centrifugation (Sigma, Osterode am Harz, Germany) at 9,000*g* at 4°C for 15 min.

Fermentations were performed in 500-ml Erlenmeyer flasks (60 ml working volume) containing non-detoxified fourfold or sixfold concentrated hydrolysates or detoxified sixfold concentrated hydrolysates. Fourfold concentrated hydrolysates were supplemented to a final concentration of 3 g l^{-1} YE or 5 g l^{-1} PEPT or 3 g l^{-1} ME or 5 g l^{-1} CAS or 3 g l^{-1} CSL. Non-detoxified and detoxified sixfold concentrated hydrolysates were supplemented with 3 g l^{-1} YE or trace elements, vitamins and minerals (TEVM) as described for the growth experiments. Detoxified hydrolysates were also supplemented with 4.5 g l^{-1} YE. Again, for all xylitol fermentations, negative controls were performed replacing the nutritive supplements volume by sterile water.

The inocula were added to reach an initial cell concentration of approximately 3 and 8 g l^{-1} (dry weight) for fourfold and sixfold concentrated media, respectively. Incubation was performed as described above. At pre-determined fermentation times, samples were withdrawn for HPLC analysis and cell growth measurements. Each cultivation was repeated at least twice and the mean values are reported.

Analytical methods

Glucose, xylose, acetic acid, formic acid, ethanol, HMF and furfural were analysed by HPLC (Waters, Milford, USA) using an Aminex HPX−87H column (Bio-Rad, Hercules, CA, USA) as described before [3]. Analyses of arabinose, xylitol, arabitol and glycerol were performed using an HPLC system (Merck, Darmstadt, Germany) equipped with a Sugar-Pak I column (Waters, Milford, USA) under the conditions described elsewhere [3].

The total amount of phenolics in the hydrolysates was determined spectrophotometrically with the Prussian blue method [13] using tannic acid as the standard.

Phosphate concentration in the hydrolysates was determined by the method described by Rouser et al. [37].

All samples were filtered through 0.45 μm filters prior to the analysis.

Cell growth was evaluated by measuring absorbance at 600 nm. At the beginning and at the end of fermentations, biomass dry weight was determined gravimetrically. The samples (5 ml) were vacuum filtered through 0.45 μm dried membrane filters (Gelman), washed with two (for non-concentrated media) or three (for concentrated media) volumes of ultra-pure water and dried overnight at 100°C to constant weight.

Calculations

The volumetric consumption rates (g l^{-1} h^{-1}) of monosaccharides (Q_S), pentoses (Q_{XylAra}) and xylose (Q_{Xyl}) were calculated as the total monosaccharides (glucose, xylose and arabinose), pentoses (xylose and arabinose) and xylose consumed in a defined time interval. The biomass (Q_X) and metabolites (Q_{XOH}, Q_{ArOH}, $Q_{Polyols}$) volumetric production rates (g l^{-1} h^{-1}), hereafter referred to as the biomass and xylitol, arabitol and total polyols productivities, were calculated as the increase in cell mass and metabolites concentrations, respectively, for a designated time interval. Total polyols are referred to xylitol and arabitol. The biomass yield, Y_{XS} (g g^{-1}), was calculated as the ratio between Q_X and Q_S. The xylitol (Y_{XOH}) and total polyols ($Y_{polyols}$) yields (g g^{-1}) were calculated as the ratios between Q_{XOH} and Q_{Xyl}, and between $Q_{Polyols}$ and Q_{XylAra}, respectively. Polyols/P is the ratio of the concentration of the total polyols produced divided by the sum of the concentration of all extracellular metabolites (xylitol, arabitol, ethanol and glycerol).

Chemicals

Granular activated charcoal (ca. 2.5 mm) and tannic acid were obtained from Merck (Darmstadt, Germany).

ME and PEPT were obtained from Oxoid (Basingstoke, England). YE was purchased from Panreac (Barcelona, Spain) and CAS from Difco (Detroit, MI, USA). CSL was kindly provided by a local starch manufacturer (Copam, S. João da Talha, Portugal).

Results and discussion

Composition of brewery's spent grain hydrolysates

Table 1 shows the composition of BSG hydrolysates used in media preparation for *D. hansenii* growth and xylitol production. The concentration step increased the monosaccharides content, and removed all furfural. It also enabled a significant removal of acetic acid and, to a lesser extent, HMF, although the effective concentration of these compounds had increased. Conversely, phenolic compounds were poorly removed, which is in agreement with previous reports [3, 19].

In sixfold concentrated hydrolysates, the detoxification with activated charcoal led to a further significant reduction in the contents of aliphatic acids and phenolic compounds, which reached final concentrations similar to or even lower compared to the fourfold concentrated hydrolysate. The high effectiveness for phenolic compounds removal owing to activated charcoal treatment has already been reported [3, 22, 26].

The total monosaccharides concentration for the sixfold concentrated hydrolysates ranged from 191.4 to 194.0 g l^{-1}, which is, to our knowledge, among the higher concentrations reported in hemicellulosic hydrolysates used for xylitol production by yeasts.

Table 1 Composition of non-concentrated and concentrated BSG hydrolysates (g l^{-1})

Compounds	Concentration factor[a]			
	1	4	6	6[b]
Xylose	16.6	54.5	108.9	109.1
Arabinose	6.76	18.3	36.7	37.8
Glucose	6.00	20.9	45.8	47.1
Acetic acid	1.50	3.90	7.23	4.66
Formic acid	0.57	2.11	4.82	2.62
Furfural	0.42	ND	ND	ND
HMF	0.06	0.08	0.39	ND
Total phenolics	1.37	3.70	9.18	2.60

ND Not detected
[a]Approximated mass concentration factor
[b]Detoxified through activated charcoal prior to the concentration

Optimisation of hydrolysate supplementation for biomass production

Effect of trace elements, vitamins and minerals

Debaryomyces hansenii can grow in BSG hydrolysate without any previous detoxification treatment or supplementation, although growth is markedly increased when the hydrolysate is supplemented with TEVM [3]. To further develop an industrial growth medium suitable for the first step of the xylitol bioprocess, it is therefore important to reduce the costs associated with hydrolysate supplementation.

Table 2 shows the effects on yeast growth of each studied nutrient group: TEVM, as well as of nitrogen, phosphate and magnesium sources per se. Supplementation with trace elements or vitamins did not change biomass productivity but decreased the level of sugar consumption. This explains the 1.7- and 1.4-fold increase in biomass yield that was observed, as compared to the non-supplemented hydrolysate. On the other hand, the simultaneous supplementation with three minerals (N, P and Mg) improved biomass productivity and yield to values similar to those obtained in the fully supplemented hydrolysate. These results could be explained by the presence of enough trace elements and vitamins for growth in the hydrolysate. Conversely, some minerals are growth limiting. The importance of phosphate and nitrogen supplementation has been demonstrated for the growth of *C. guilliermondii* [30] and *C. blankii* [20] in sugarcane bagasse hydrolysate.

Considering the effects of the individual minerals, the phosphate source was, by far, the most important. Phosphate supplementation led to similar biomass productivity as obtained with the mixture of the three

Table 2 Effect of supplementation on biomass production by *D. hansenii* in BSG hydrolysate

Supplementation	Glc	Xyl	Ara	Q_S (g l^{-1} h^{-1})	Q_X (g l^{-1} h^{-1})	Y_{XS} (g g^{-1})
	(Percentage consumed)					
None	74	13	5	0.21	0.05	0.23
TEVM	100	100	85	0.75	0.48	0.64
TE	64	4	4	0.14	0.05	0.33
V	57	8	5	0.13	0.05	0.38
M (N, P and Mg)	100	96	95	0.77	0.53	0.69
N	65	0	0	0.10	0.05	0.51
P	100	95	96	0.76	0.55	0.73
Mg	63	0	0	0.10	0.07	0.76

Glc Glucose; *Xyl* xylose; *Ara* arabinose; Q_S volumetric monosaccharides consumption rate; Q_X biomass productivity; Y_{XS}, biomass yield; *TEVM* (mixture of *TE* trace elements, *V* vitamins, and *M* minerals); *N* nitrogen source; *P* phosphate source, *Mg* magnesium source

minerals and even a slight increase in biomass yield was observed. Furthermore, the level of sugar consumption was not affected when the hydrolysate was supplemented only with the phosphate source. In contrast, the nitrogen and magnesium sources per se did not improve yeast growth. Phosphate supplementation of hydrolysates is necessary to balance the phosphate removal that occurs during the neutralisation of acid hydrolysates [15, 30]. We found that after neutralisation about half of the phosphate was lost during sterile filtration (data not shown).

Hydrolysate by-products, such as acetic acid and formic acid, were fully consumed only when phosphate was supplemented, whereas furfural was completely exhausted in all conditions. The high biomass yields attained in this work are similar to those previously reported for this yeast [4], and can be partially explained by the assimilation of extra carbon derived from the by-products [4, 11, 24].

Effect of phosphate concentration

Since phosphate was identified as the growth-limiting nutrient, the minimum concentration of KH_2PO_4 required to reach the highest cell growth was studied (Fig. 1). The final biomass concentration increased with the increase of the KH_2PO_4 concentration up to 0.5 g l^{-1}, but remained quite stable at higher concentrations. A similar pattern was obtained for biomass yield (data not shown). Since the results obtained were similar to those obtained in BSG hydrolysate supplemented with the three minerals (Table 2), a concentration of 0.5 g l^{-1} KH_2PO_4 was selected for further use during the inoculum preparation for xylitol production trials.

Under these optimised conditions, yeast displayed a maximum specific growth rate, biomass yield and productivity of 0.34 h^{-1}, 0.60 g g^{-1} and 0.55 g l^{-1} h^{-1}, respectively. These data are similar to the values previously reported for this yeast in BSG hemicellulosic hydrolysates [3] but are higher than those reported for hydrolysate-grown *C. tropicalis* for a similar initial xylose concentration [30, 31]. Indeed, the biomass productivity obtained in this work is only surpassed by values obtained under more oxygenated conditions, e.g., using a bioreactor [18, 24].

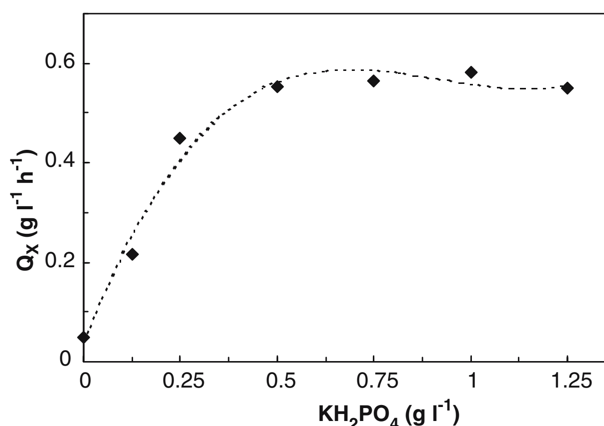

Fig. 1 Influence of KH_2PO_4 concentration on *D. hansenii* biomass productivity in BSG hydrolysate

Under the studied conditions no metabolic products other than CO_2 and biomass were identified, which may further explain the high biomass yield and productivity obtained. This is an advantage over other yeasts, which even under aerobic conditions may produce extracellular compounds, such as ethanol [17].

Validation of growth media composition

To ensure that the sole phosphate-supplemented BSG hydrolysate can indeed fully support *D. hansenii* growth, the yeast cells obtained after one biomass production cycle were further used as inoculum for another cultivation cycle for a total cycle number of four. The results obtained for the final biomass concentration and yield were fairly similar for all batches, varying less than 10% among the different cycles, demonstrating that yeast was able to reach the same growth performance (data not shown). This is of biotechnological interest since it is possible to produce biomass with a high efficiency in a potentially low-cost medium.

Effect of hydrolysates supplementation on xylitol production

The effects of several complex nutrients, such as ME, PEPT, CAS, YE and CSL, on the bioconversion of non-detoxified (fourfold concentrated) BSG hydrolysate to xylitol by *D. hansenii* were studied (Table 3). As an example, Fig. 2 shows the kinetic profile of *D. hansenii* when grown in YE-supplemented BSG hydrolysate.

In all conditions, glucose was completely depleted between 24 and 28 h of fermentation time and its consumption rate was almost not affected by supplementation (data not shown). Xylose consumption was increased for all tested supplements, with YE producing the highest value. Xylose and glucose were consumed simultaneously although the hexose was consumed at a higher rate. After glucose exhaustion, the xylose consumption rate only occasionally surpassed the rate previously obtained during the simultaneous consumption of both sugars (data not shown). Arabinose consumption started when xylose reached the arabinose concentration, and at the end of fermentation, the total arabinose consumption varied between 20% (for non-supplemented and ME-supplemented media) and 95% (for YE and CSL-supplemented media). The simultaneous consumption of xylose and arabinose differs from that reported for *C. guilliermondii*, for which the arabinose consumption starts only after xylose exhaustion [1]. Furthermore, the arabinose consumption rates were

Table 3 Effect of supplementation on kinetic and stoichiometric parameters of *D. hansenii* growth and polyols formation in non-detoxified fourfold concentrated BSG hydrolysates

Supplementation	Glc	Xyl	Ara	Q_X (g l^{-1} h^{-1})	Q_{XOH} (g l^{-1} h^{-1})	Y_{XOH} (g g^{-1})	Q_{ArOH} (g l^{-1} h^{-1})	$Y_{Polyols}$ (g g^{-1})	Polyols/P
	(% consumed)								
None	100	62	5	0.08	0.04	0.08	0.20	0.50	0.80
TEVM[a]	100	82	29	0.28	0.29	0.51	0.10	0.59	0.94
CAS	100	80	6	0.14	0.09	0.16	0.27	0.60	0.89
CSL	100	70	23	0.29	0.23	0.43	0.16	0.67	1.00
ME	100	74	11	0.08	0.15	0.27	0.19	0.59	0.84
PEPT	100	74	12	0.10	0.08	0.14	0.22	0.51	0.83
YE	100	90	15	0.26	0.36	0.55	0.14	0.72	0.97

All parameters were calculated after 72 h, except Q_X that was calculated at the end of the fermentation run

CAS Casamino acids; *CSL* corn steep liquor; *ME* malt extract; *PEPT* peptone; *YE* yeast extract; Q_{XOH} xylitol productivity; Q_{ArOH} arabitol productivity; Y_{XOH}, xylitol yield; $Y_{Polyols}$ polyols yield; *Polyols/P* ratio of total polyols/total extracellular metabolites

[a]Ref. [3]

always lower than those for xylose, which is in agreement with the previous reports for *D. hansenii* [3] and other xylitol-producing yeasts [6, 16, 21].

Xylitol and arabitol production was clearly favoured in all conditions, as reflected by the ratio of total polyols/total extracellular metabolites. Arabitol was produced from both xylose and arabinose. Conversely, xylitol seems to be solely produced from xylose metabolism. Polyols production in non-supplemented BSG hydrolysates suggested that some essential nutrients are already present, as previously described for rice straw hydrolysate [35]. Ethanol and glycerol were also produced, but only in small amounts.

Supplementation increased pentose consumption and a concomitant increase in polyols and biomass production was also observed (Table 3). Arabitol production was favoured in CAS- and PEPT-supplemented media whereas similar levels of xylitol and arabitol were attained in ME-supplemented media, suggesting that the stress conditions that might have induced arabitol production could not be fully relieved by those supplements. In contrast, xylitol production was clearly favoured for TEVM-, CSL- and YE-supplemented media. These supplements not only induced an increase in xylitol production, but also decreased the arabitol production rate. The highest xylitol/arabitol ratio (2.6) was obtained for YE-supplemented media. Compared to the non-supplemented media, the polyols yield increased by approximately 1.4-fold. This is in agreement with previous results in which YE has been described to increase xylitol production both in synthetic xylose-based media [14, 21] and in hemicellulosic hydrolysates [32].

At the fermentation time corresponding to the maximum xylitol concentration, the xylose consumption ranged from 62 to 90%, whereas the arabinose consumption was far less (Table 3). The lowest consumption of both pentoses occurred in non-supplemented media, stressing the importance of supplementation. Xylitol yield and productivity increased according to the following increasing order: PEPT < CAS < ME < CSL < TEVM < YE.

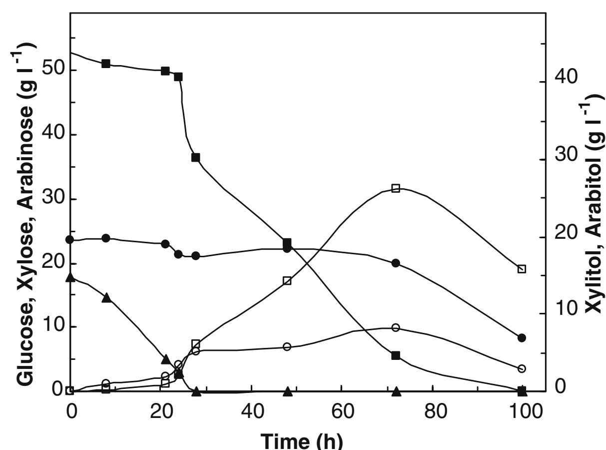

Fig. 2 Profiles of monosaccharides consumption and polyols production by *D. hansenii* in non-detoxified fourfold concentrated BSG hydrolysate supplemented with yeast extract. Glucose (*filled triangle*), xylose (*filled square*), arabinose (*filled circle*), xylitol (*open square*), arabitol (*open circle*)

Effect of hydrolysates concentration and detoxification on xylitol production

The concentration degree of the hydrolysates is of major importance for xylitol production since it affects both the xylose and the inhibitor concentrations in the media. These factors, together with supplementation, are among the major factors to be considered during the optimisation of xylitol bioprocess using hemicellulosic hydrolysates. Table 4 shows the results obtained for *D. hansenii* when cultivated in non-supplemented or supplemented sixfold concentrated BSG hydrolysate with or without previous detoxification.

Compared to the fourfold concentrated hydrolysates, one of the most remarkable effects of increasing hydrolysate concentration is the decrease in polyols productivity for all non-detoxified supplemented media. In fact, xylitol productivity was reduced, except for non-supplemented hydrolysate, whereas arabitol productivity was rather insensitive to concentration or supplementation.

Table 4 Effect of detoxification and supplementation on polyols formation by *D. hansenii* in sixfold concentrated BSG hydrolysates

Supplementation	Detoxification[a]	Glc	Xyl	Ara	Q_{XOH} (g l^{-1} h^{-1})	Y_{XOH} (g g^{-1})	Q_{ArOH} (g l^{-1} h^{-1})	$Y_{Polyols}$ (g g^{-1})	Polyols/P
		(% consumed)							
None	No	100	93	15	0.12	0.26	0.11	0.46	0.93
None	Yes	100	65	8	0.07	0.23	0.09	0.51	0.85
TEVM	No	100	90	8	0.27	0.65	0.06	0.78	0.91
TEVM	Yes	100	62	24	0.11	0.39	0.05	0.56	0.83
YE 3	No	100	95	30	0.16	0.38	0.12	0.60	0.95
YE 3	Yes	100	62	3	0.07	0.24	0.07	0.47	0.82
YE 4.5	Yes	100	65	8	0.08	0.26	0.07	0.46	0.80

YE 3 Yeast extract concentration of 3 g l^{-1}; *YE 4.5* yeast extract concentration of 4.5 g l^{-1}
[a]Activated charcoal treatment

Furthermore, the higher xylitol and arabitol productivities were always obtained in non-detoxified media regardless of supplementation and despite both aliphatic acid levels and the especially high phenolic compounds concentration.

A second important effect is the increase in xylitol and total polyol yields found for non-detoxified TEVM-supplemented hydrolysates. They showed the highest yields of xylitol and polyols attained in this work. Xylitol and polyol yields were not improved by detoxification, regardless of supplementation.

The best xylitol (and total polyol) productivities obtained in the present work were found for fourfold concentrated hydrolysates-based media (Table 3), which contained the lower concentration of aliphatic acids but a somewhat high concentration of phenolic compounds (Table 1). In fact, although phenolic compounds have been considered to exert a significant inhibition on the fermentation of lignocellulosic hydrolysates, some recent reports state that the highest xylitol productivities and/or yields do not occur for detoxification treatments that led to the highest removal of phenolic compounds [3, 23]. Moreover, there has been evidence that some phenolics, namely hydroquinone, significantly increased xylose consumption and xylitol production by *D. hansenii* [12].

The monosaccharides assimilation pattern is similar to that described for fourfold, with two exceptions: 4.5 g l^{-1} YE-supplementation increased the initial xylose consumption rate above the glucose consumption rate and after glucose exhaustion the xylose consumption rate increased in all media (1.7- to 3.3-fold of the initial rates). As an example, Fig. 3 shows the kinetic profile of *D. hansenii* when grown in detoxified TEVM-supplemented hydrolysate. Xylitol accumulation rates increased proportionally with the increase of xylose consumption. On the contrary, arabitol concentrations increased steadily from the beginning until the maximum was reached. Glycerol and ethanol production reached higher concentrations than those observed in the fourfold concentrated media. *D. hansenii* usually produces glycerol and arabitol as a response to stress [7]. Glycerol can be produced from both glucose and xylose and the increase in glycerol production observed for higher concentrated hydrolysates could reflect an augmented stress situation.

The xylose concentration in the sixfold concentrated hydrolysates (around 100 g l^{-1}) was near the optimum value for maximizing xylitol production previously described for this yeast in synthetic xylose medium [36]. The xylitol yield obtained in this work (hydrolysate-based medium) was even higher compared to the yield obtained from xylose [36]. This work shows that *D. hansenii* tolerates simultaneously high concentrations of sugars (total content around 190 g l^{-1}) and inhibitors (total content of acetic acid, formic acid, furfural, HMF and phenolics of the media was around 20 g l^{-1}). A careful optimisation of the oxygen availability is thus of major importance to further optimise xylitol productivity from BSG hydrolysates, given that a significant interaction between oxygen and medium composition is foreseen [36].

Conclusions

The minimum supplement requirement for efficient biomass production by *D. hansenii* in BSG hemicellulosic

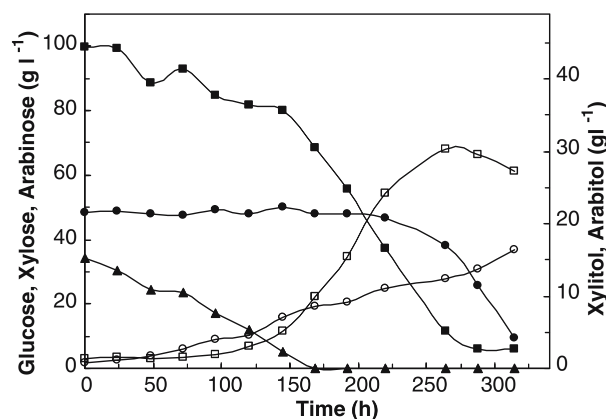

Fig. 3 Profiles of monosaccharides consumption and polyols production by *D. hansenii* in sixfold concentrated BSG hydrolysate detoxified with activated charcoal and supplemented with trace elements, vitamins and minerals. Glucose (*filled triangle*), xylose (*filled square*), arabinose (*filled circle*), xylitol (*open square*), arabitol (*open circle*)

hydrolysate was identified as only 0.5 g l^{-1} KH_2PO_4. Furthermore, the yeast cells obtained after a cultivation run when used consecutively as inoculum in this medium were able to reach the same growth performance. This has significant advantages at the industrial level.

The supplementation of fourfold concentrated BSG hydrolysates increased the xylitol (and total polyols) yield and productivity. The increase in xylitol productivity and yield could be related to the increase in the xylose consumption rate induced by supplementation. YE was found to be the best of the tested supplements.

A further increase in the hydrolysate concentration factor led to a decrease in xylitol (and total polyols) productivity. Conversely, an increase of xylose concentration is still important to enhance xylitol yield. The detoxification of the latter hydrolysate did not improve the xylitol production even when the supplements concentration was increased, suggesting that the factors limiting xylitol production are not related to the toxic effect of inhibitors but are due to some other unidentified nutritional limitation, e.g., oxygen.

This work clearly demonstrated that *D. hansenii* can tolerate high concentrations of monosaccharides and inhibitors and is able to produce polyols as the major extracellular metabolites from BSG hydrolysates, even without any nutrient supplementation.

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