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Xylitol Production by *Debaryomyces hansenii* in Extracted Olive Pomace Dilute-Acid Hydrolysate

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Abstract: The extracted olive pomace (EOP) is an industrial lignocellulosic by-product of olive pomace oil extraction, currently mainly used for energy production through combustion. In this work, the hemicellulosic fraction of EOP was selectively hydrolyzed by diluted acid hydrolysis to obtain pentose-rich hydrolysates that can potentially be upgraded by *Debaryomyces hansenii*, targeting xylitol production. The monosaccharides and degradation by-products released along the pre-treatment were quantified and several detoxification methods for the removal of potentially toxic compounds were evaluated, including pH adjustment to 5.5, the use of anion-exchange resins, adsorption into activated charcoal, concentration by evaporation, and membrane techniques, i.e., nanofiltration. The latter approach was shown to be the best method allowing the full removal of furfural, 41% of 5-hydroxymethylfurfural, 54% of acetic acid, and 67% of the phenolic compounds present in the hydrolysate. The effects of the supplementation of both non-detoxified and detoxified hydrolysates were also assessed. The non-detoxified hydrolysate, under aerobic conditions, supported the yeast growth and xylitol production at low levels. Supplementation with the low-cost corn steep liquor of the nanofiltration detoxified hydrolysate showed a higher xylitol yield (0.57 g/g) compared to the non-detoxified hydrolysate. The highest xylitol productivity was found in hydrolysate detoxified with anionic resins (0.30 g/L·h), which was 80% higher than in the non-detoxified culture medium. Overall, the results showed that EOP dilute acid hydrolysates can efficiently be used for xylitol production by *D. hansenii* if detoxification, and supplementation, even with low-cost supplements, are performed.

Keywords: *Debaryomyces hansenii*; detoxification; extracted olive pomace; pentose-rich hydrolysate; supplementation

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1. Introduction

The olive oil industry is a relevant sector for the south European countries, which is responsible for 67% of the world's olive oil production [1]. The olive oil production generates a semi-solid by-product (olive pomace), which can be used for the extraction of olive pomace oil together with the production of a subsequent lignocellulosic residue called extracted olive pomace (EOP). EOP is a hemicellulose-rich agro-industrial by-product from which hemicelluloses can be fractionated by chemical or biological hydrolysis allowing the production of sugars (mainly pentoses), which can be still converted to valuable products,

e.g., xylitol [2]. Several pre-treatment technologies [3] are commonly used, among which dilute-acid hydrolysis is one of the simplest and fastest methods for the hydrolysis of this biomass [4]. Acidic hemicellulosic hydrolysates comprise a mixture of sugars and contain in its composition a considerable complex mixture of aliphatic acids (with acetic acid as the major constituent), furan derivatives (furfural and 5-hydroxymethylfurfural, HMF), and phenolic compounds. However, mild dilute acid processes have the advantage of potentially producing hydrolysates with a higher sugar/inhibitors ratio [5,6].

To facilitate further conversion, namely by fermentation, several approaches are being considered for the removal of those inhibitors. The most common detoxification approaches include chemical or physico-chemical treatments such as pH neutralization, sulphite treatment, overliming, adsorption into activated charcoal, and treatment with anionic resins [7]. Besides these, membrane-based processes also hold a promising potential [8]. As hydrolysate detoxification influences the economy of the process, the development of efficient and inexpensive detoxification methods is needed. Furthermore, the detoxification methods must be optimized for each process since the chemical composition of the hydrolysate also depends on the species, their age, and the origin of biomass.

Xylitol is a low-calorie sugar alcohol with a sweetness power similar to sucrose. It is an interesting compound due to its anticarcinogenic and cariostatic properties, enhancer of tooth remineralization, negative heat of solution (pleasant cool and fresh sensation), and adequacy for diabetics as a glucose replacer [2]. Furthermore, it is a building block and a top value-added chemical [9] with significant potential. Due to its properties and applications, xylitol is widely used as a food additive and oral health ingredient, presenting an average market price of 5000 to 20,000 USD/ton [10], depending on the targeted application specifications. Although xylitol can be found naturally in fruits and vegetables, its extraction from natural sources is not economically viable due to its low concentrations. Therefore, the global demand for this ingredient (approximately 125,000 tons in 2020 and expected to grow in the next years) is largely supported by chemical production. Nevertheless, the chemical xylitol production, which is based upon catalytic xylose dehydrogenation, offers some drawbacks such as the required high purity of the feedstock xylose solution, removal of the catalyst, as well as, the use of high temperatures and pressures making it a high-cost process, not only for being energy-intensive but also environmentally risky due to the use of toxic catalysts and high-pressure hydrogen gas [11].

As an alternative to the chemical methods biotechnological processes are also being studied. The production of xylitol using bacteria, fungi, and yeasts has been researched for decades, but yeasts are recognized as the best xylitol producers. Most studied yeast belong to the genus *Candida* (mainly *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*), as well as yeasts such as *Debaryomyces hansenii*, which have been described as xylitol-overproducers [5,12]. These processes have the advantage of allowing the decrease of the environmental impact and energy requirements [13].

To be efficiently converted, detoxification and hydrolysate supplementation are the two major factors influencing medium suitability for xylitol bioproduction, and the use of low-cost supplements is always a requirement to keep production costs viable.

In the present work, the diluted acid hydrolysis of the EOP was studied. The hydrolysates obtained were subjected to several detoxification treatments such as pH adjustment, concentration by evaporation, activated charcoal adsorption, treatment with anionic resins, and membrane (nanofiltration) techniques. The hydrolysate fermentability was evaluated by comparing the xylitol formation in both non-detoxified and detoxified hydrolysates. Additionally, hydrolysate supplementation was also studied. Thus, the influence of fermentation medium pH, oxygen availability, and different types of supplements with trace elements, vitamins, and minerals (TEVM), yeast extract (YE), corn steep liquor (CSL), and brewery's spent grain extract (BSGE) on yeast performance and xylitol bioproduction was evaluated.

2. Material and Methods

2.1. Raw Material

The extracted olive pomace (EOP) used in this work was provided by UCASUL (Alvito, Beja, Portugal). The EOP was sieved to retain particles between 1.00 and 3.55 mm, homogenized into a defined lot, and stored at room temperature in plastic containers. The remaining particles were discarded. The chemical composition of the EOP contained on a dry solid basis (*w/w*) 23.7% hemicellulose (of which 17.7% xylan, 1.2 arabinan, 4.8% acetyl groups) and 16.8% cellulose, which makes up the total carbohydrate content of 40.5%, 37.9% lignin, and 14.9% of extractives.

2.2. Preparation and Treatment of EOP Hydrolysate

The feedstock was mixed with 3.5% (*w/w*) sulfuric acid solution in closed flasks at a solid-to-liquid ratio of 5 (g H_2SO_4/g dry feedstock). The hydrolysis was carried out at previously optimized conditions at 130 °C for 130 min [8] in an autoclave (Uniclave, Portugal). After the reaction, the hydrolysate and solid residue were recovered by pressing (up to 200 bar) using a hydraulic press (Sotel, Portugal). The collected hydrolysate was filtered through filter paper (Filter-Lab 1235, Barcelona, Spain) and chemically characterized as described below. The hydrolysate was stored at 4 °C for later use.

2.3. Detoxification Procedures

The detoxification treatments were carried out over raw or pH-adjusted hydrolysates. After the detoxification, all detoxified hydrolysates were concentrated (two-fold) and the pH was adjusted to 5.5, 6.5, or 7.5 by addition of the NaOH pellets.

2.3.1. Activated Charcoal Treatment

The granulated charcoal (ca. 2.5 mm) (Merck, Germany) was sequentially washed with water and adjusted to a pH 7 with 0.4 M HCl, washed with ultra-pure water, and dried for 48 h at room temperature. The charcoal detoxification of hydrolysates was carried out by mixing charcoal and hydrolysates at a pH equal to 2.5 (1:10 (*w/v*)) and stirring for 1 h at room temperature according to a procedure published elsewhere [14]. The detoxified hydrolysate was recovered by vacuum filtration and the pH value was adjusted to 5.5, 6.5, and 7.5 with solid NaOH.

2.3.2. Treatment with Anionic-Exchange Resins

The weak anion-exchange resins (Marathon, Las Vegas, NV, USA) (Sigma-Aldrich, St. Louis, MO, USA) were equilibrated with 0.1 M NaOH solution, washed with ultra-pure water, and finally filtered until an effluent with neutral pH was obtained were used in this study. The equilibrated resins were separated by vacuum filtration using filter paper (Filter-Lab 1235) and dried at room temperature for 48 h. The resins were added to the hydrolysate until pH reached 5.5 or 6.5 and the mixture was stirred for 1 h at room temperature. The treated hydrolysate was recovered by vacuum filtration.

2.3.3. Adsorptive Membranes (Nanofiltration)

The raw hydrolysate was treated with adsorptive membranes NF270 (Dow, Midland, MI, USA) (nanofiltration) at continuous operation mode and constant feed volume, using a transmembrane pressure of 20 bar and pH 3.0. The full procedure of the nanofiltration used in this work was previously described elsewhere [8].

2.3.4. Evaporation

The raw hydrolysates were concentrated by evaporation to remove about 25% of the initial weight of hydrolysate at a pH of 5.5 and 50% of hydrolysates at pH 5.5, 6.5, and 7.5, respectively. The evaporation treatment was performed using a rotatory evaporation system (Büchi, Flawil, Switzerland) at 150 mbar, and 70 °C.

2.4. Microorganism and Inoculum Conditions

For inocula preparation, 2.5 mL of *Debaryomyces hansenii* CCMI 941 stock cultures (stored at −70 °C) were used to inoculate 1000 mL baffled Erlenmeyer flasks containing 80 mL of chemically defined medium [12]. The yeast cells were grown in an orbital incubator (Infor® Unitron, Bottmingen, Switzerland) at 30 °C and 150 rpm. After 22 h of growth, 40 mL of cells were harvested by sterile centrifugation (10 min, 4 °C, 8035× *g*) (Sigma 3K15 centrifuge, Osterode am Harz, Germany) and resuspended in 1000 mL Erlenmeyer flasks containing sterile hydrolysate using 0.22 µm filters (Millipore, Bedford, MA, USA).

2.5. Medium and Fermentation Conditions

The fermentations were carried out in 1 L Erlenmeyer flasks containing 72.8 mL of fermentation medium and an initial cell concentration of 2.5 g/L (dry weight). The flasks were maintained in the same growth conditions as described above. To evaluate the oxygen availability effect on xylitol production different Erlenmeyer flasks were used. In low and high oxygen availability conditions, unbaffled and baffled Erlenmeyer flasks were used, respectively. The fermentation media were supplemented with trace elements, vitamins, and minerals (TEVM), yeast extract (YE) (Sigma, St. Louis, MO, USA) (3 g/L), corn steep liquor (CSL, Copam, Portugal) (5 g/L), or brewery spent grain extract (BSGE) (0.5 g/L).

2.6. Analytical Methods

Monosaccharides (glucose, xylose, and arabinose), acetic acid, 5-hydroxymethylfurfural (HMF), and furfural present in hydrolysates were analysed by HPLC (Agilent 1100 series HPLC system, Santa Clara, CA, USA) using the Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) in combination with a cation H^+-guard column (Bio-Rad). The detection was made using a refractive index (RI) and an ultraviolet detector (UV). The elution took place at 50 °C with 5 mmol/L H_2SO_4 at a 0.6 mL/min flow rate. Sugars and acetic acid were detected by the RI detector; furfural and HMF were detected by the UV detector at 280 nm. Owing to the partial overlap of arabinose, xylitol, and arabitol, those components were also analyzed by HPLC using a Sugar-Pak I column (Waters, Milfort, MA, USA) operating at 90 °C, in a Merck system (Merck, Darmstadt, Germany) equipped with a RI detector. The mobile phase was 50 mg/L calcium EDTA with a 0.5 mL/min flow rate. Total phenolic compounds were determined by the Folin-Ciocalteu colourimetric method [15]. In short, 100 µL of the sample was mixed with 5 mL of the 1/10 (*v/v*) diluted Folin-Ciocalteu reagent and 4 mL of 7.5% (*w/v*) Na_2CO_3. An absorbance was measured at 765 nm after 15 min of incubation at 45 °C. Total phenolic compound concentrations were expressed in mg of gallic acid equivalent per mL of solution (mg GAE/mL). Fermentation samples were analysed for monosaccharides, aliphatic acids, furan derivatives, ethanol, and glycerol, using the HPLC methods described above. All samples were filtered through 0.45 µm membrane filters (Millipore, USA) before the HPLC analysis. The cell growth was evaluated by measuring the absorbance at 600 nm using an LKB spectrophotometer (Biochrom, Cambridge, UK). At the beginning and end of fermentation cell biomass dry weight was determined gravimetrically as described elsewhere [14].

The xylose-to-xylitol bioconversion yield ($Y_{P/S}$) (g/g) was the ratio between xylitol production (g/L) and xylose consumption (g/L). The productivity (Q_P) (g/L.h) was determined by the ratio between xylitol production (g/L) and fermentation time (h).

3. Results and Discussion

3.1. EOP Hydrolysate Composition

The first step of the present study consisted of the preparation of the EOP hemicellulosic hydrolysate. As shown in Table 1, the EOP hydrolysate contained 36.9 g/L of total fermentable monosaccharides among which xylose was the sugar released in the highest concentration, reaching a maximal content of 32.6 g/L. Glucose and arabinose were found in lower concentrations, respectively 1.98 g/L and 2.30 g/L, showing that the concentration of pentose sugars is more than seventeen-fold higher than glucose. In

addition to sugars, compounds potentially harmful to the fermentation process, such as acid acetic (from the deacetylation of xylan) and furan dehydration products (furfural and hydroxymethylfurfural) and phenolic compounds were also found (Table 1).

Table 1. Composition of the extracted olive pomace hydrolysate produced by dilute-acid hydrolysis, after pH adjustment to 5.5.

Component	Concentration [a] (g/L)
Xylose	32.6
Arabinose	1.98
Glucose	2.30
Acetic acid	9.10
HMF	0.06
Furfural	2.66
Phenolic compounds	5.02

[a] Deviations < 5%.

Phenolic compounds were also found in relatively high concentrations but in lower amounts than acetic acid. Phenolic compounds are formed as products of partial dissolution of lignin fraction and/or could be present in the raw material as extractives and are easily soluble in water. It is noteworthy that phenolic compounds present in olive pomace namely hydroxytyrosol, tyrosol, oleuropein, caffeic acid, *p*-coumaric, vanillic acid, catechol, and rutin are easily water-extractable compounds [16,17].

Furfural was the main furan derivative found and HMF was only detected in trace amounts. Apart from the fact that under dilute-acid hydrolysis conditions, pentoses are usually more susceptible to degradation than hexoses, pentose sugars (namely xylose) are produced in higher amounts and thus furfural is found in higher concentrations than the HMF. The high xylose concentration present in the hydrolysate definitively favours the bioconversion to produce xylitol. Nevertheless, the amount of acetic acid and phenolic compounds together with some furfural imposes their removal to make bioconversion possible. Moreover, the high concentration of toxic compounds obtained may be ascribed to the high solid loading, which was possible to be used for this material, as well as both the high acetyl and extractives content in the raw material. Therefore, the employment of detoxification treatment before the conversion process is envisaged as highly recommended.

3.2. Effect of Detoxification on the Chemical Composition of the Hydrolysate

One of the main difficulties in the bioconversion of sugars from lignocellulosic hydrolysates into value-added products is the negative effect of degradation compounds formed during the hydrolysis step. Several approaches (detoxification methods) have been developed to reduce the amounts of these toxic compounds, but, in general, detoxification also reduces sugar concentration in hydrolysates. Because of this, and as high xylose concentration is relevant for xylitol production, hydrolysates detoxified by activated charcoal, anionic resins, and nanofiltration were subject to a further concentration (2×) process (Table 2). On the other hand, concentration by evaporation may also be employed as a detoxification method as shown later in this work. The sugars and toxic compounds removal observed for each treatment is shown in Table 2. The pH adjustment to 5.5 aims to correct the acidity to be adequate for microbial activity and therefore it cannot be considered as an effective method for reduction of inhibitory compounds. However, some reduction of furans occurs, and all detoxification treatments applied decreased efficiently the furan derivatives content.

Table 2. Effect of detoxification treatment on the composition of extracted olive pomace. Results are expressed as a percentage of the compound's mass left after treatment or after treatment and after concentration.

Detoxification Treatment	Monosac. [a]	Acetic Acid	HMF	Furfural	Phenolics [b]
None	100	100	100	100	100
pH 5.5	93	95	74	59	94
Concentration (1.5×)	87	87	93	5	84
Concentration (2×)	88	83	84	0	90
Activated charcoal	99	89	38	13	27
Activated charcoal + conc. (2×)	99	54	27	44	52
Anionic resins	99	99	74	37	41
Anionic resins + conc. (2×)	100	72	47	0	78
Nanofiltration	90	46	59	0	33
Nanofiltration + conc. (2×)	84	41	69	0	83

[a] Monosac.: total monosaccharides; [b] Phenolics.: total phenolics.

The concentration of hydrolysates is an important step for bioprocess efficiency because it enables the increase of sugar concentration in a culture medium and can be used as a detoxification treatment. In EOP hydrolysates the evaporation enables to reduction of the furans derivatives content, in particular furfural. Furfural, as a volatile compound, can be easily removed by evaporation; therefore, it was eliminated after concentration (2×). The obtained results are in agreement with the data presented in previous reports [5,14]. The HMF concentration was significantly reduced by the activated charcoal treatment (62%) followed by the concentration step (73%). Also, the treatment with anionic resins has been reported as an efficient method of the reduction of furan derivatives in spruce hydrolysates and in brewery's spent grain [14,18]. In contrast to the furfural, the acetic acid removal was lower. The highest reduction of this aliphatic acid was obtained by nanofiltration (54%) followed by activated charcoal treatment (11%). The first, when combined with evaporation allowed to reduce aliphatic acid concentration by 59%. It is worth observing that although acetic acid is a volatile compound it is very difficult to remove and due to its high amounts, it constitutes the most important toxic fraction in the hydrolysate. Removal of more than 30% of acetic acid just using anionic resins (Dowex Marathon WBA) was reported in a brewery's spent grain hydrolysate [14]. A concentration (2×) of hydrolysate led to the removal of 17% of acetic acid although the removal degree depends on the extent of evaporation. Another interesting aspect observed was that all the detoxification methods applied enabled a total phenolic compound reduction. However, the most pronounced reduction (73%) was obtained with activated charcoal, and it was similar to 76% or higher (58%) than reported earlier for sugarcane bagasse hydrolysate and brewery's spent hydrolysates, respectively [19,20]. As it was mentioned before, the removal of phenolic compounds is a very important step for the fermentative process and additionally, phenolics e.g., hydroxytyrosol are value-added compounds per se. Comparing the treatments used, it can be concluded that the highest reduction of toxic compounds was achieved with nanofiltration and activated charcoal. It is important to highlight that even low levels of toxic compounds may inhibit the bioconversion process. It might be due to the synergetic effect of such compounds that may still constrain the fermentation process [21]. Besides the significant reduction of degradation compounds, detoxification also results in a considerable drop in the sugar concentration. Sugar removal was observed for all the tested conditions, although some differences were observed. The nanofiltration treatment allowed a decrease of total monosaccharides close to 10% while the evaporation (1.5×) allowed a reduction of 13% of total monosaccharides. This data can be elucidated by the formation of some precipitates after the concentration step. Other authors found similar results when evaluating the detoxification of rice straw hemicellulosic hydrolysate using activated carbon adsorption and observed that the xylose concentrations were reduced by 4% to 18% [22].

3.3. Effect of Detoxification on Xylitol Production

To evaluate the degree of microbial growth inhibition caused by the EOP hydrolysate, *D. hansenii* was grown in both detoxified- and non-detoxified hydrolysate (only subjected to pH correction). The composition of the EOP hydrolysates used in xylitol production experiments is shown in Table 3. The differences found in hydrolysates composition are a consequence of the detoxification methodology applied and of the mass concentration factor (1.5 or/and 2×) achieved as all detoxified hydrolysates were concentrated by evaporation to increase the xylose content which potentially favours xylitol bioproduction.

Table 3. Composition (g/L) of EOP hydrolysate (after pH adjustment, detoxification, and concentration) for xylitol production experiments.

Hydrolysate Treatment	pH	Xyl	Ara	Glc	Acetic Acid	Furfural	HMF	Phenolics
Control	5.5	23.5	2.54	1.60	7.75	1.85	0.05	4.11
Concentration (1.5×)	5.5	32.6	3.52	2.31	9.33	0.15	0.05	3.45
Concentration (2×)	5.5	56.9	4.94	4.30	11.9	n.d	0.06	3.87
Concentration (2×)	6.5	56.1	4.92	4.28	10.9	n.d	0.05	3.90
Concentration (2×)	7.5	55.6	4.91	4.15	10.7	n.d	0.05	4.02
Activated charcoal + conc. (2×)	5.5 [a]	40.9	4.71	4.69	9.80	n.d	0.02	1.90
Activated charcoal + conc. (2×)	6.5 [a]	39.8	4.62	4.69	9.78	n.d	0.02	2.10
Activated charcoal + conc. (2×)	7.5 [a]	39.4	4.39	4.48	9.76	n.d	0.02	3.00
Anionic resins + conc. (2×)	5.5	42.6	3.50	2.70	9.33	n.d	0.03	4.20
Anionic resins + conc. (2×)	6.5 [a]	31.8	3.24	3.24	10.3	n.d	0.02	4.70
Nanofiltration + conc. (2×)	5.5	51.7	5.23	3.97	6.18	n.d	0.05	3.50

[a] hydrolysates sterilized by autoclaving; n.d, non-detected.; Xyl, xylose; Ara, Arabinose; Glc, Glucose.

3.3.1. Evaporation

The results of the fermentation assays using the concentrated hydrolysates are shown in Figure 1.

As observed in detoxification assays, the concentration of acetic acid in hydrolysates increased after concentration by evaporation. Considering the effect of pH on acetic acid toxicity, the pH of concentrated (2×) hydrolysates was adjusted to 5.5, 6.5 and 7.5 and the pH of non-concentrated and concentrated (1.5×) hydrolysates was adjusted to 5.5 to evaluate this effect on xylitol production. The non-concentrated hydrolysate was used as a control assay.

All the xylitol production experiments were performed in high oxygen availability conditions. For both non-concentrated and concentrated (2×) hydrolysates, a longer lag phase was observed as compared to what was obtained in concentrated (1.5×) hydrolysate. This data indicates that the conversion of the xylose into xylitol by *D. hansenii* was influenced by the extension of the concentration procedure.

More specifically, the concentration (1.5×) procedure essentially allowed the reduction of furfural content in the culture medium allowing simultaneously the yeast growth more efficiently. On the other hand, the concentration (2×) allowed the complete removal of furfural but also led to an increase of acetic acid concentration in the culture medium. The increase of the acetic acid is very harmful to the yeast metabolism, once in the concentrated (2×) hydrolysate at pH 5.5 no yeast growth was observed. For all hydrolysates glucose and xylose were consumed simultaneously being glucose firstly depleted. In non-concentrated hydrolysate, xylose consumption increased after 40 h of fermentation, and in the concentrated (1.5×) hydrolysate xylose was consumed after 72 h. The most concentrated (2×) hydrolysates showed a distinct xylose consumption profile, e.g., after 72 h fermentation, 35% and 70% of xylose consumption was observed in the concentrated hydrolysate at pH of 6.5 and 7.5, respectively. At the same time, arabinose consumption was observed. *D. hansenii* did not show the diauxic consumption of glucose and xylose a behaviour that was already reported in other hydrolysates [5]. Regarding arabinose, a maximum consumption of 66% and 73% in non-concentrated and concentrated hydrolysates was observed,

respectively. The xylitol was the only product released during fermentation and neither ethanol nor glycerol was detected. The highest xylitol concentration, 11.2 g/L was obtained in concentrated (2×) hydrolysate at pH 6.5 (Table 4). For this hydrolysate, the xylitol yield and productivity were 30% and 25% higher than the values obtained in non-concentrated hydrolysate. However, a higher volumetric productivity (0.19 g/L.h) was attained for concentrated (1.5×) hydrolysate, due to the significant removal of furfural and increase of sugars in hydrolysate. This data shows that the growth limitation in concentrated and non-concentrated hydrolysates seems to be the high concentration of inhibitor compounds in this hydrolysate, namely, acetic acid, suggesting the need for an efficient detoxification methodology.

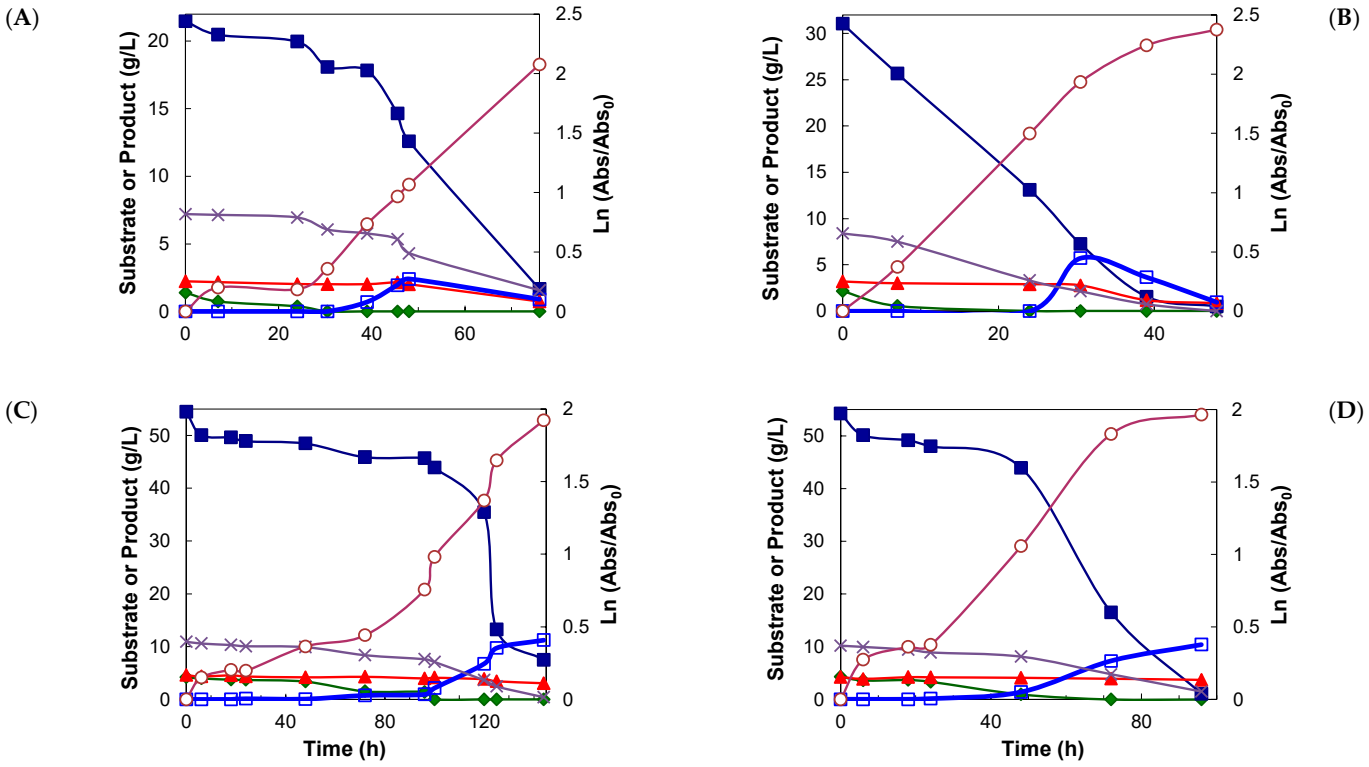

Figure 1. Effect of EOP dilute-acid hydrolysate detoxification by concentration (1.5 and 2×) on xylitol production by *D. hansenii* CCMI 941. Non-detoxified hydrolysate (pH = 5.5) (**A**); hydrolysate concentrated (1.5×, pH = 5.5) (**B**); hydrolysate concentrated (2×, pH = 6.5) (**C**); hydrolysate concentrated (2×, pH = 7.5) (**D**). xylose (■); xylitol (□); arabinose (▲); glucose (◆); acetic acid (×); cell density (○).

3.3.2. Activated Charcoal Adsorption

The xylitol production in activated charcoal detoxified and concentrated (2×) hydrolysates was also studied (Figure 2). In addition, the effect of initial pH (5.5, 6.5, and 7.5) and oxygen availability was also assessed.

As in the previous experiments, relatively long lag phases were observed, except for hydrolysate at pH 7.5. In that case, the short lag phase can be explained by the fact that acetic acid undergoes more extensive dissociation (pK_a = 4.76) and thus is less toxic for yeast metabolism. As occurred in previous assays, and for all hydrolysates, glucose, and xylose were consumed simultaneously being glucose firstly depleted. The rate of sugar consumption changes with the pH conditions. At pH 6.5 and with high oxygen availability, total glucose consumption occurred after 45–72 h. The total consumption of xylose was also observed for all hydrolysates except for those at pH 6.5 with high oxygen availability (92% consumption after 76 h of fermentation). Arabinose consumption was only 53% and 16% in detoxified hydrolysates at pH 7.5 and 5.5, respectively.

Table 4. Kinetic and stoichiometric parameters of *D. hansenii* CCMI 941 on product formation in extracted olive pomace hydrolysates.

Detoxification Treatment	pH	Oxygen Availability	Supplements	X_0 (g/L)	Xyl_0 (g/L)	Xyl (%)	Y_{XOH} (g/g)	Q_{XOH} (g/L/h)	Q_x (g/L/h)
Control	5.5	High	TEVM	0.9	21.5	78.0	0.17	0.06	0.15
Evaporation (1.5×)	5.5	High	TEVM	1.6	31.0	98.4	0.19	0.19	0.39
Evaporation (2×)	5.5	High	TEVM	2.5	54.8	-	-	-	-
Evaporation (2×)	6.5	High	TEVM	1.0	54.5	86.3	0.24	0.08	0.15
Evaporation (2×)	7.5	High	TEVM	1.8	54.3	98.0	0.20	0.13	0.15
Activated charcoal	5.5	High	TEVM	3.2	39.1	69.6	0.21	0.13	0.18
Activated charcoal	6.5	High	TEVM	4.6	38.6	92.2	0.17	0.10	0.28
Activated charcoal	6.5	Low	TEVM	4.2	36.8	99.9	0.22	0.13	0.25
Activated charcoal	7.5	High	TEVM	2.6	36.8	83.2	0.16	0.11	0.27
Anionic resins	5.5	Low	TEVM	3.2	45.8	83.9	0.42	0.30	0.09
Anionic resins	6.5	Low	TEVM	2.3	28.7	83.6	0.45	0.20	0.12
Nanofiltration	5.5	Low	TEVM	2.2	48.8	90.8	0.39	0.14	0.07
Nanofiltration	5.5	Low	YE	2.2	46.4	92.9	0.55	0.17	0.04
Nanofiltration	5.5	Low	CSL	2.1	49.9	92.5	0.57	0.19	0.06
Nanofiltration	5.5	Low	BSGE	1.5	50.7	92.7	0.47	0.16	0.05

X_0, initial biomass concentration; Xyl_0, initial xylose concentration; Xyl, xylose consumed; Y_{XOH}, xylitol yield on xylose consumed; Q_{XOH}, xylitol production rate; Q_x, cell productivity; YE, yeast extract; CSL, corn steep liquor; BSGE, brewery's spent grain extract. All parameters were calculated at the maximum xylitol concentration; Low: Unbaffled flasks; High: Baffled flasks.

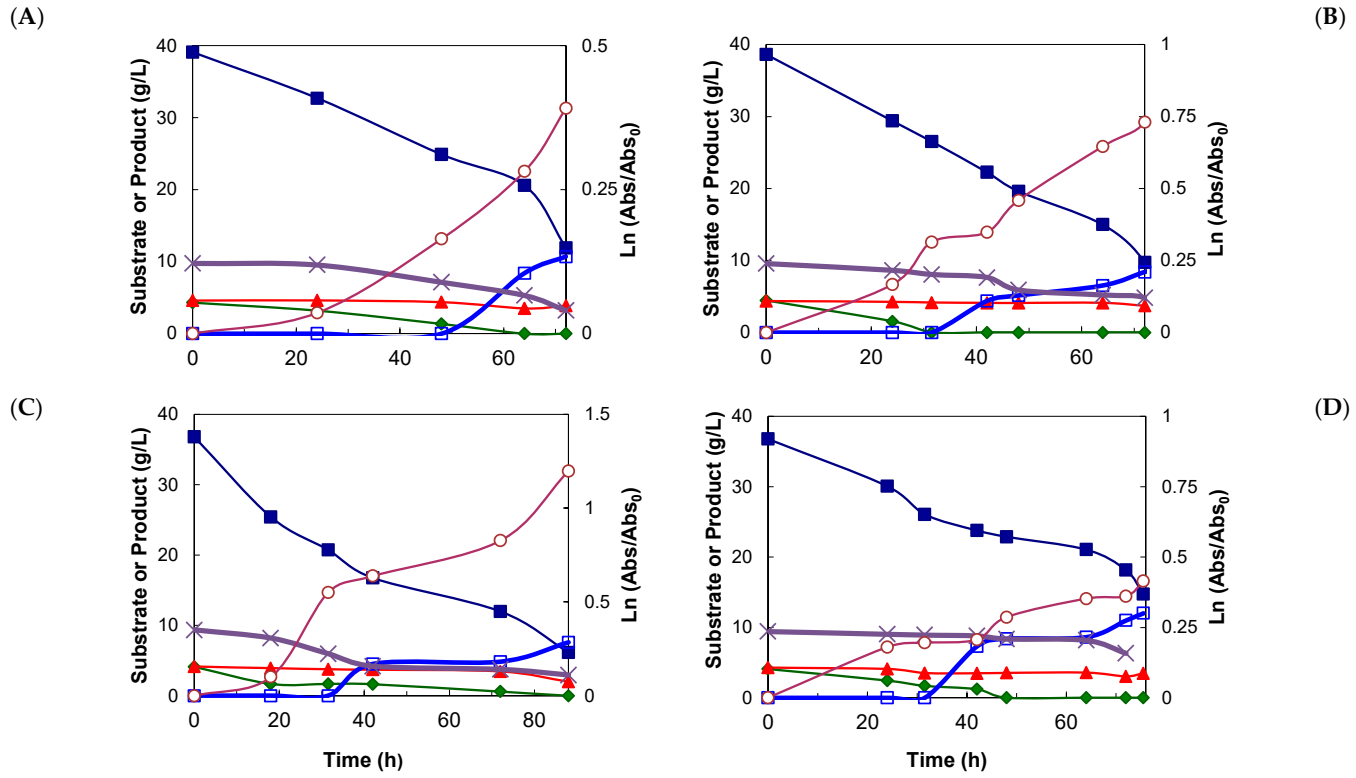

Figure 2. Effect of EOP dilute-acid hydrolysate detoxification with activated charcoal followed by concentration (on xylitol production by *D. hansenii* CCMI 941. Concentrated hydrolysate (2×, pH = 5.5) (**A**); detoxified and concentrated hydrolysate (2×, pH = 6.5), high oxygen availability (**B**); detoxified and concentrated hydrolysate (2×, pH = 6.5), low oxygen availability (**C**); detoxified and concentrated hydrolysate (2×, pH = 7.5), high oxygen availability (**D**). xylose (■); xylitol (□); arabinose (▲); glucose (◆); acetic acid (×); cell density (○).

The highest xylitol yield and productivity were obtained for hydrolysate at pH 6.5 (Table 4) with low oxygen availability. Nevertheless, it is noteworthy that the data obtained are very similar to those obtained in hydrolysates at pH 5.5 with higher oxygen availability. The comparison of both assays at pH 6.5 allowed us to conclude that higher oxygen availability favours yeast growth instead of xylitol production. This fact is demonstrated by the 23% increase in xylitol yield and productivity for pH 6.5 hydrolysates with low oxygen availability as compared to hydrolysate with high oxygen availability. As previously emphasized, the assays with hydrolysates at different pHs allowed to support that to obtain higher xylitol productivity and yield, the pH value must be as close as possible to the optimal value of microbial growth. The highest xylitol yield was attained for non-detoxified and concentrated (2×) hydrolysates at pH 6.5, which means that activated charcoal treatment negatively affected xylitol production. These data can be associated with changes in medium viscosity, which are visible after detoxification and that could hamper microbial growth and xylitol production. After the detoxification, a colourless hydrolysate was obtained, indicating that efficient detoxification had occurred, and this was confirmed by chemical analysis. These data, in general, agree with other studies. For example, experiments with sorghum hydrolysate show similar xylitol productivity but yield decreases significantly when detoxified with activated charcoal [23]. However, other studies showed improvements in the xylose consumption and xylitol productivity, for example when a chestnut shell or eucalyptus hydrolysates were subject to an activated charcoal detoxification methodology [24,25]. These differences, namely the decrease in the xylitol productivity and yield when a detoxified hydrolysate is tested, can also probably be associated with some sugar loss due to the detoxification procedure.

3.3.3. Anionic Resins

The effect of detoxification with anionic resins and hydrolysate pH on the xylitol production was also studied. In this study, a hydrolysate with pH 7.5 was not evaluated once previous experiments showed that this pH does not favour high xylitol yield and productivity. In this case, some changes in the medium viscosity during pH correction to 6.5 were observed, leading to the need for medium sterilization by autoclaving. All experiments were performed in low oxygen availability conditions. The data obtained is shown in Figure 3. In this case, a loss of 23% in total monosaccharides was observed, reducing the yeast performance. Contrary to the previous experiments, the lag phase was very short for both hydrolysates. In pH 5.5 hydrolysate a high increase in biomass production was observed compared to hydrolysate at pH 6.5. In this study, the high acetic acid concentration did not show a strong inhibitory effect even at the highest concentration (9 g/L). For hydrolysates at pH 5.5 and pH 6.5 its consumption was not significant, 13% and 21%, respectively. The glucose and xylose were consumed simultaneously, although the glucose was completely consumed within the first 24 h while 16% of the xylose was still found after 67 h. The arabinose consumption was similar and was as high as 15% for both experiments. The highest xylitol concentration, 16.3 g/L, was achieved in the case of hydrolysate at pH 5.5 (Table 3). At this condition, the xylitol productivity was 27% higher than in hydrolysate at pH 6.5. Nevertheless, the maximum xylitol yield was attained with slightly acidic hydrolysate (pH 6.5). All achieved data allowed us to conclude that to obtain optimal xylitol production, a xylose concentration close to 50 g/L and limited oxygen availability are necessary. The results obtained favourably compared to those obtained in olive pruning hydrolysates. Martin et al., 2010 and Cuevas et al., 2009 [26,27] reported a lower xylitol yield (0.13 g/g) for *Candida tropicalis* grown in olive tree pruning hydrolysates or *Pachysolen tannophilus* in olive pits hydrolysates, respectively [26,27]. Despite the apparent high toxicity of the EOP hydrolysate at pH 5.5 the obtained xylitol productivity and yield are 17 and 13% higher than that previously obtained in anionic resin detoxified brewery spent grain hydrolysate [14]. Our data also favourably compare to those obtained by López-Linares [28] with the yeast *Candida boidinii* grown in exhausted olive

pomace hemicellulosic hydrolysate, detoxified with ion-exchange resins, which reported a maximum xylitol yield of 0.43 g/g.

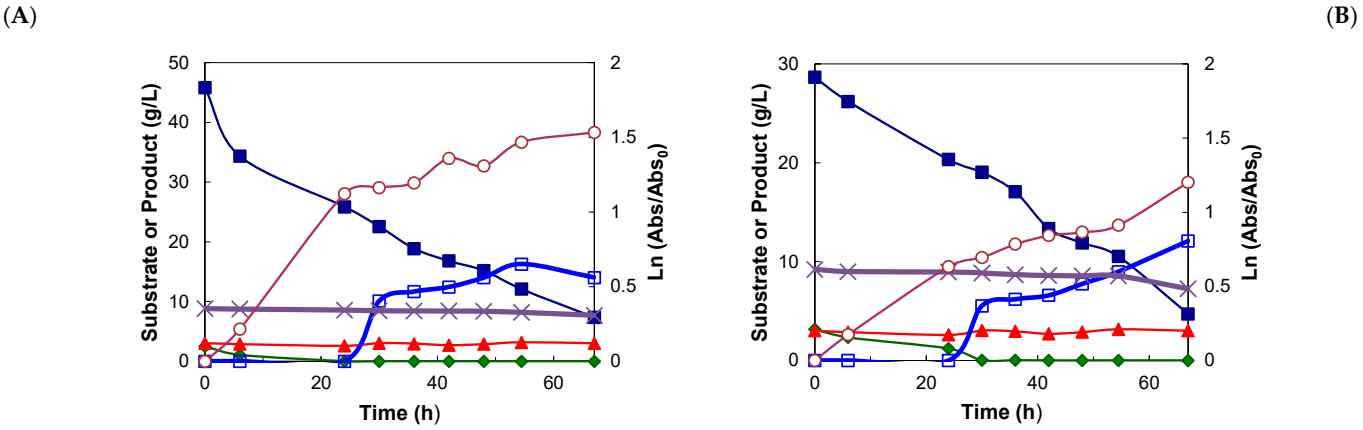

Figure 3. Effect of EOP dilute-acid hydrolysate detoxification with anionic resins followed by concentration on xylitol production by *D. hansenii* CCMI 941. Detoxified and concentrated hydrolysate (2×, pH = 5.5) (**A**); detoxified and concentrated hydrolysate (2×, pH = 6.5) (**B**); xylose (■); xylitol (□); arabinose (▲); glucose (◆); acetic acid (×); cell density (○).

3.3.4. Nanofiltration and Supplementation

After detoxification, supplementation is also a major factor that affects yeast performance and it is always required, at some level, to increase the production at competitive costs. Figure 4 shows the effect of the various supplements (yeast extract, corn steep liquor, TEVM and brewery's spent grains extract, BSGE) on xylitol production.

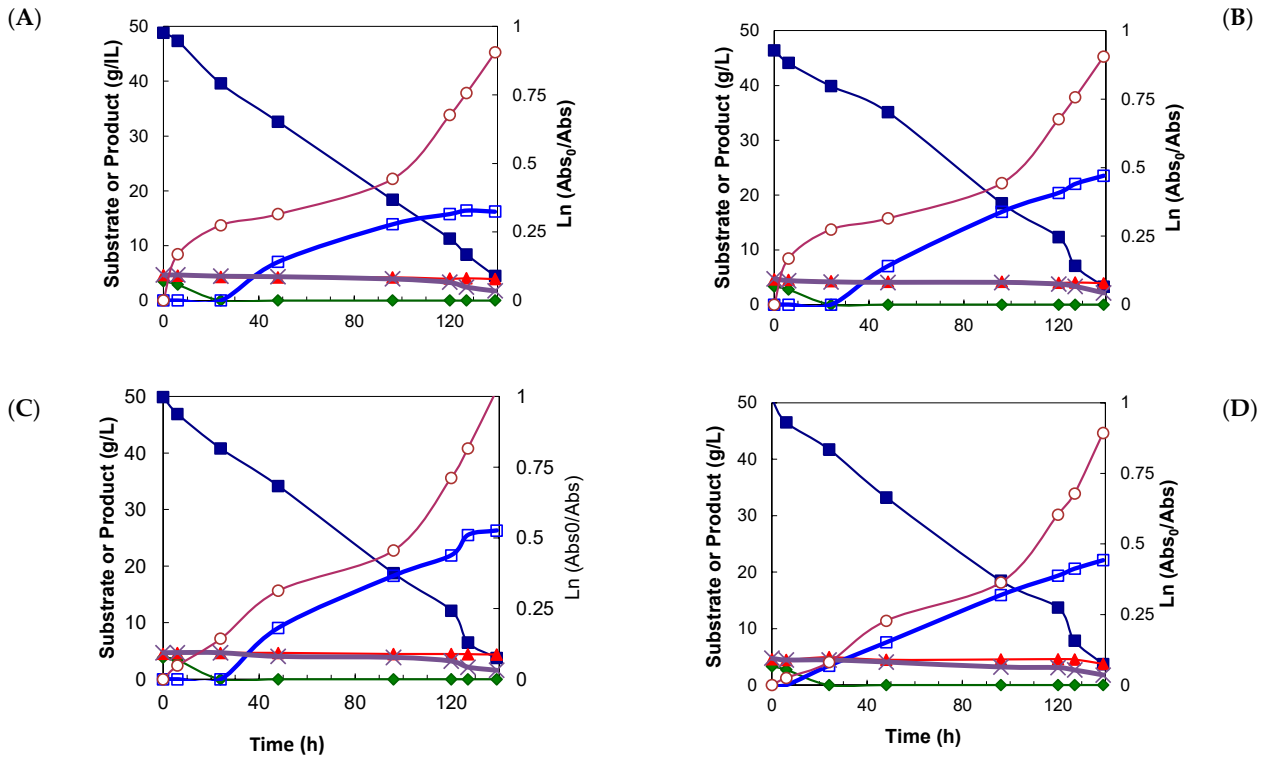

Figure 4. Effect of EOP dilute-acid hydrolysate detoxification by nanofiltration followed by concentration on xylitol production by *D. hansenii* CCMI 941 in: TEVM supplemented hydrolysate (**A**); yeast extract supplemented hydrolysate (**B**); CSL supplemented hydrolysate (**C**); and BSGE supplemented hydrolysate (**D**); xylose (■); xylitol (□); arabinose (▲); glucose (◆); acetic acid (×); cell density (○).

The hydrolysates detoxified by nanofiltration were concentrated (2×) by evaporation and pH was adjusted to 5.5. As a comparison with other hydrolysates subjected to different detoxification methodologies, the hydrolysate supplemented with TEVM was used as a control. The yeast showed efficient growth in all hydrolysates, with a short lag phase. This can be due to the absence of HMF and furfural, together with a lower content of acid acetic (4.66 g/L). Acetic acid was consumed during the fermentation assay. Its consumption was 54% in the hydrolysate supplemented with yeast extract and 62–66% for the remaining hydrolysates. The overall sugar consumption rates depend on the supplement. Glucose and xylose were consumed simultaneously and as in previous experiments, glucose was the first sugar consumed (within 12 h). The xylose consumption rate was the same for all hydrolysates (0.30 g/L.h) except for hydrolysate supplemented with BSGE in which the xylose consumption rate was 0.34 g/L.h. As for the other experiments, *D. hansenii* did not present a diauxic consumption of glucose and xylose, although the xylose consumption rate did increase after glucose exhaustion. The arabinose consumption ranged between 4% and 18% after 138 h. To assess the effect of nanofiltration as a detoxification methodology in xylitol production the stoichiometric parameters obtained in the hydrolysate supplemented with TEVM were calculated and compared. Among all TEVM-supplemented hydrolysates, the highest xylitol concentration (17.51 g/L) was obtained in hydrolysate detoxified by nanofiltration. However, this condition did not allow us to obtain the highest xylitol productivity which was 53% lower than the obtained in the hydrolysate detoxified with anionic resins (pH 5.5). This data suggests that the complete removal of furan derivatives may have a negative effect on xylitol production. The studies carried out with *Candida magnolia* showed that furfural is a competitive inhibitor of growth. Nevertheless, the presence of a certain amount of furfural (∼0.3 g/L) in the production medium improved both the productivity and yield of xylitol under suitable oxygen-limited conditions [29].

For all hydrolysates, xylitol was the major product and neither ethanol nor glycerol was detected. Supplementation influenced the stoichiometric parameters. The highest xylitol concentration (26.3 g/L) was obtained with 5 g/L CSL supplementation. The xylitol yield and volumetric productivity were respectively 32% and 26% higher than the values attained for the control medium (supplementation with TEVM). The CSL has been previously reported to play an important role in the bioconversion process [5,30]. The xylitol productivities and yields reported in this paper are similar to those described for *D. hansenii* NRRL Y-7426 in concentrated eucalypt hydrolysate containing 80 g/L xylose [31]. Yeast extract (3 g/L) favoured xylitol productivity for the EOP hydrolysates, being 28% higher than the values attained for the control assay. The BSGE (0.5 g/L) obtained from hydrothermal treatment of brewery spent grains allowed to obtain xylitol productivity 17% higher than the control assay. Therefore, the CSL and yeast extract were shown to be the best supplements. However, considering the low cost of commercial CSL, this supplement emerged as an alternative to conventional and expensive supplements, although it is important to define the specific composition of the supplement and identify which compounds are responsible for the increase of both xylitol yield and productivity. Despite the high level of potentially toxic compounds present in the OEP hydrolysates, the data obtained in this study, after detoxification and low-cost supplementation also favourably compared to previous results obtained for a different *D. hansenii* strain (NRRL Y-7426) and were also similar to those obtained with *C. guilliermondii* in rapeseed straw hemicellulosic hydrolysate [32].

4. Conclusions

The overall results obtained for the xylitol production by *D. hansenii* CCMI 941 in the concentrated EOP hemicellulosic hydrolysates showed that detoxification has an important impact on both xylitol yield and productivity, as both were significantly improved by detoxification treatments by nanofiltration or by anionic resins. The other detoxification treatments resulted only in a slight increase in both parameters. Supplementation is the second major factor influencing medium fitness, being xylitol production improved by

supplementation. The best results were attained for the hydrolysate supplemented with 5 g corn steep liquor/L which has the advantage of being a low-cost supplement, a relevant requirement to keep production costs manageable.

The data attained, especially the xylitol yields achieved in resins- or nanofiltration-detoxified hydrolysates, favourably compared with many others reported in the literature. These data demonstrate that the EOP, a potentially difficult substrate for bioconversion purposes, has a strong potential to be used in the biotechnological production of xylitol by *D. hansenii*. Future studies should rely on the optimization of supplement concentration and yeast adaptation to the hydrolysates.

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