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Biovalorization of Lignocellulosic Materials for Xylitol Production by the Yeast *Komagataella pastoris*

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Abstract: The main goal of this study was to screen different lignocellulosic materials for their ability to support the cell growth of the yeast *Komagataella pastoris* and the production of xylitol. Several lignocellulosic materials, namely banana peels, brewer's spent grains (BSGs), corncobs, grape pomace, grape stalks, and sawdust, were subjected to dilute acid hydrolysis to obtain sugar rich solutions that were tested as feedstocks for the cultivation of *K. pastoris*. Although the culture was able to grow in all the tested hydrolysates, a higher biomass concentration was obtained for banana peels (15.18 ± 0.33 g/L) and grape stalks (14.58 ± 0.19 g/L), while the highest xylitol production (1.51 ± 0.07 g/L) was reached for the BSG hydrolysate with a xylitol yield of 0.66 ± 0.39 g/g. Cell growth and xylitol production from BSG were improved by detoxifying the hydrolysate using activated charcoal, resulting in a fourfold increase of the biomass production, while xylitol production was improved to 3.97 ± 0.10 g/L. Moreover, concomitant with arabinose consumption, arabitol synthesis was noticed, reaching a maximum concentration of 0.82 ± 0.05 g/L with a yield on arabinose of 0.60 ± 0.11 g/g. These results demonstrate the feasibility of using lignocellulosic waste, especially BSG, as feedstock for the cultivation of *K. pastoris* and the coproduction of xylitol and arabitol. Additionally, it demonstrates the use of *K. pastoris* as a suitable microorganism to integrate a zero-waste biorefinery, transforming lignocellulosic waste into two high-value specialty chemicals with high market demand.

Keywords: xylitol; arabitol; lignocellulose; hydrolysis; *Komagataella pastoris*

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1. Introduction

Xylitol is a naturally occurring pentahydroxy sugar alcohol used as a natural food sweetener [1,2]. It can be found in a wide variety of fruits, vegetables, berries, oats, and mushrooms, in amounts of less than 1 wt% [3]. This polyol has sweetness equivalent to sucrose but with a lower glycemic index, which makes it suitable for diabetes patients [4,5]. Due to its anticariogenic properties, xylitol has also been used in the prevention of caries; it is a common component of sugar-free chewing gums [6,7]. Additionally, the nutritional benefits of xylitol promote its use in several dietary supplements [8,9], nutraceuticals [10,11], and confectionary products [12]. Owing to its trend across food and pharmaceutical industries, in 2019 the xylitol global market exceeded 880 million USD, and it is further expected to expand to over 1 billion USD by 2026 [13].

On an industrial scale, xylitol is produced though the catalytic hydrogenation of xylose from xylose-enriched biomass, such as birch wood, in a high energy-consuming and a nonenvironmentally friendly process [3]. Over the last decades, the biotechnological pathway has emerged as an ecological alternative for xylitol production because it involves milder conditions, low carbon emissions, and lower environmental disposal of chemicals [14]. Moreover, the use of lignocellulosic waste as a feedstock for microbial cultivation makes biotechnological processes economically sustainable [15,16].

Various xylose-rich lignocellulosic waste, including corncobs [17], sugarcane bagasse [18], brewer's spent grain (BSG) [19], and rice straw [20], have been used as feedstock for the

microbial production of xylitol. Due to their complex structure, lignocellulosic materials generally require pretreatment steps, such as dilute acid hydrolysis, to convert cellulose and hemicellulose into fermentable sugars, prior to their utilization as feedstocks for microbial cultivation. However, while maximizing the conversion of the carbohydrates into fermentable sugars, it is essential to minimize the carbohydrates losses during processing to ensure the cost-effectiveness of the pretreatment [21]. Such treatments can remarkably modify the physical–chemical properties of biomass and generate inhibitors by degradation of the formed products. Although it is a simple, rapid, and low-cost method, dilute acid hydrolysis generates several toxic compounds, namely furan derivatives and weak acids, which can act as microbial inhibitors, leading to slow kinetics, limited yields, and low productivities [15,22]. In view of this, several detoxification methods, such as evaporation, neutralization, overliming, adsorption into ion-exchange resins, or activated charcoal, have been used to remove toxic compounds or reduce their concentration, thus increasing the hydrolysates' fermentability [23,24]. The utilization of activated charcoal has arisen as a simple low-cost method for hydrolysates' detoxification, and its use significantly improved microbial cell growth as well as xylitol synthesis [25,26].

Yeasts, especially those belonging to the genus *Candida* [27–29] that possess the enzymes required for xylose metabolism, are recognized as the most efficient xylitol producers [15]. However, the pathogenic nature of *Candida* sp. is a barrier for the bioprocess implementation at an industrial scale, due to safety issues. An alternative is the use of genetically modified xylitol-producing yeasts (e.g., *Saccharomyces cerevisiae* XP-RTK [30], *Pichia stipitis* YS-30 [31]), but the legislation related with genetically modified organisms (GMOs) products also restricts their industrial use [32]. Therefore, the utilization of nonpathogenic and non-GMO microorganisms for the production of xylitol represents a promising strategy to overcome such issues and develop novel bioprocesses for this sugar alcohol production. *Komagataella pastoris* (formerly known as *Pichia pastoris*), a wild-type and generally recognized as safe (GRAS) yeast, demonstrated the ability to grow and produce xylitol using glucose/xylose mixtures as feedstock [33]. Although in the study xylose was not utilized by the cells for cell growth, which was based on the assimilation of glucose, the culture efficiently utilized xylose for the synthesis of xylitol.

The aim of this study is to evaluate the viability of using different lignocellulosic materials as feedstocks to produce xylitol by *K. pastoris*. Several lignocellulosic materials are subjected to dilute acid hydrolysis, and the resulting hydrolysates are tested as the sole feedstock for the cultivation of *K. pastoris* in batch shake flask assays. To improve cell growth and xylitol synthesis, the raw BSG hydrolysate was concentrated and detoxified by treatment with activated charcoal. The impact of the treated hydrolysate was assessed in terms of cell growth, xylitol synthesis, and xylitol yield on a substrate basis.

2. Materials and Methods

2.1. Raw Materials

Banana peels were mashed and preserved in sealed bags at −20 °C, until further use. BSG was supplied by Sociedade Central de Cervejas (Vialonga, Portugal), dried at 70 °C until constant weight, ground in a mill, and stored in sealed bags at room temperature. Corncobs and sawdust were collected from a local farm and a local sawmill, respectively (Carregal do Sal, Portugal), and stored in sealed bags at room temperature. Grape pomace and grape stalks were kindly provided by Quinta do Ribeiro Santo (Carregal do Sal, Portugal). Both feedstocks were dried at 70 °C until constant weight, milled, and stored in sealed bags at −20 °C, until further use.

2.2. Characterization of the Materials

The moisture content of the raw materials was determined by subjecting samples (1 g) to a temperature of 105 °C, until constant weight was attained. Dried samples were further subjected to pyrolysis at 575 °C, for 24 h, to evaluate their ash content. The particle size distribution of BSG, grape pomace, grape stalks, and sawdust, after drying at 70 °C

until constant weight and then milling, was determined by submitting samples (100 g) to a mechanical sieving shaker, using sieves with pores sizes in the range of 106 to 2360 µm, for 15 min. The fraction collected in each sieve was weighed separately. The protein content of the raw materials was evaluated by elemental analysis, using a Flash EA 1112 CHNS analyzer (Thermo Scientific, Waltham, MA, USA). The protein content was estimated by the following equation:

$$\text{Protein} = \text{N} \times 6.25, \quad (1)$$

where N represents the total nitrogen and 6.25 is a nitrogen-to-protein conversion factor [34].

To determine the hemicellulose, cellulose, and lignin contents of the materials, a well-established procedure as described by Sluiter et al. [35] was used. Briefly, samples of dried raw materials (300 mg) were hydrolyzed with sulfuric acid (3 mL H_2SO_4 72%) at 30 °C, for 1 h, under constant stirring. Afterwards, the samples were diluted with deionized water (84 mL) and further hydrolyzed in an autoclave (121 °C, 1 bar, 20 min).

After hydrolysis, the solutions were vacuum filtered, and the hydrolysis liquors obtained were used for quantification of the released monosaccharides and the acid soluble lignin (ASL). The remaining solid residues were used to quantify acid insoluble lignin (AIL), after being dried (105 °C, 4 h) and subjected to pyrolysis at 575 °C, for 24 h. The AIL (%) content was determined by the following equation:

$$\text{AIL} = ((\text{weight}_{\text{dried}} - \text{weight}_{\text{pyrolyzed}})/\text{ODW}_{\text{sample}}) \times 100 \quad (2)$$

where $\text{ODW}_{\text{sample}}$ represents the oven-dry weight (g) of each sample calculated by using the moisture content (%) described above. For determination of ASL, hydrolysis liquors samples were diluted in deionized water, and the absorbance was measured at 240 nm. Each sample was analyzed in duplicate, and the ASL (%) content was given by the following equation:

$$\text{ASL} = ((\text{abs} \times \text{V}_{\text{filtrate}} \times \text{dilution factor})/(\varepsilon \times \text{ODW}_{\text{sample}} \times \text{L})) \times 100 \quad (3)$$

where abs is the average of UV–Vis absorbance for each sample, $\text{V}_{\text{filtrate}}$ corresponds to the volume of hydrolysis liquor (L), ε represents the absorptivity of biomass at 240 nm (L/g cm), and L is the pathlength of the UV–Vis cell (cm). Depending on the raw material, the ε values used were 12 L/g cm for sawdust and grape stalks; 25 L/g cm for grape pomace, banana peels, and BSG; and 30 L/g cm for corncobs [35]. The total amount of lignin (%) was given by the sum of AIL and ASL.

For identification and quantification of the released monosaccharides, the pH of hydrolysis liquors was adjusted to values between 5 and 6 by addition of calcium hydroxide. After removal of the calcium sulphate precipitate by centrifugation (13,F, 20 min), the supernatants were used for quantification of monosaccharides by high performance liquid chromatography (HPLC), using a CarboPac PA10 and AminoTrap columns (Dionex, Thermo Scientific, Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (PAD). The samples were analyzed at 25 °C, using NaOH and CH_3COONa as the eluent, at a flow rate of 1 mL/min. Glucose (Merck KGaA, Darmstadt, Germany), xylose (Sigma-Aldrich, Darmstadt, Germany), galactose (Fluka, Buchs, Switzerland), arabinose (AppliChem Panreac, Germany), mannose (Sigma-Aldrich, Darmstadt, Germany), fructose (Scharlau, Barcelona, Spain), sucrose (Fluka, Buchs, Switzerland), and maltose (VWR, Lisboa, Portugal) were used as standards in a concentration range from 5 to 100 ppm.

Finally, the cellulose, xylan, and arabinan contents (%) were calculated by the following equations:

$$\text{Cellulose content (\%)} = ((\text{GH}/1.111)/(\text{weight}/\text{V}_{\text{filtrate}})) \times 100 \quad (4)$$

$$\text{Xylan content (\%)} = ((\text{XH}/1.136)/(\text{weight}/\text{V}_{\text{filtrate}})) \times 100 \quad (5)$$

$$\text{Arabinan content (\%)} = ((\text{AH}/1.136)/(\text{weight}/\text{V}_{\text{filtrate}})) \times 100 \quad (6)$$

where GH, XH, and AH represent glucose, xyloses and arabinose concentrations (g/L), respectively, and weight (g) is the amount of raw material used. The terms 1.111 and 1.136 are the mass conversion factors for glucose, xylose, and arabinose to cellulose, xylan, and arabinan, respectively [35]. The content of hemicellulose was estimated by the sum of xylan and arabinan.

2.3. Preparation of Hydrolysates

BSG, grape pomace, grape stalks, and sawdust were dried and milled as described above, while banana peels were mashed into a puree, and corncobs were cut into smaller pieces. The materials were then subjected to acid hydrolysis using sulfuric acid in an autoclave (121 °C, 1 bar). The hydrolysis conditions applied to each material are summarized in Table 1, which were defined based on the literature. The resulting mixtures were centrifuged (13,131× *g*, 4 °C, 30 min), and the solid fraction was discarded. Prior to fermentation, the pH value of the hydrolysates was adjusted to 5 by an addition of $Ca(OH)_2$ or $CaCO_3$, and the formed precipitate was removed by centrifugation (13,131× *g*, 4 °C, for 20 min). The liquors that were obtained, referred as raw hydrolysates, were used as cultivation media for testing their suitability in supporting cell growth and xylitol synthesis.

Table 1. Hydrolysis conditions applied to the different lignocellulosic materials.

Raw Material	Concentration of H_2SO_4 (% *w*/*w*)	Liquid to Solid Ratio (% *w*/*w*)	Time of Hydrolysis (min)	Reference
Banana peels	1	10	40	[36]
BSG	3	8	20	[37]
Corncobs	6	10	40	[17]
Grape pomace	3.3	8	30	[38,39]
Grape stalks	3	8	20	[40]
Sawdust	6	8	20	[41]

2.4. Preparation of BSG Hydrolysates

2.4.1. Concentration of the BSG Hydrolysates

The raw BSG hydrolysate obtained as described above was concentrated (twofold) by placing the solution in an oven at a temperature of 70 °C, for 24 h, for solvent evaporation.

2.4.2. Detoxification Treatment

Detoxification of the concentrated hydrolysate was performed as described by Kamal et al. [42], with slight modifications. Briefly, powdered activated charcoal (Sigma-Aldrich, Darmstadt, Germany) was mixed with BSG concentrated hydrolysates at a concentration of 2.5% (*w*/*v*) and magnetic stirred (800 rpm), for 1 h, at room temperature. The activated charcoal was removed by centrifugation (13,131× *g*, 30 min at 4 °C), and the detoxified BSG hydrolysates were used as cultivation medium.

2.5. Characterization of Hydrolysates

The sugar composition of the raw hydrolysates and the treated BSG hydrolysates was determined by HPLC, as described above.

The hydrolysis efficiency, i.e., the conversion of cellulose, xylan, and arabinan into glucose, xylose, and arabinose (represented as GluR, XylR and AraR, respectively), were obtained using the following equations (adapted from [37]):

$$\text{GluR (\%)} = 0.90 \times ((\text{C}_{\text{sugar}} \times \text{V})/(\text{cellulose content} \times \text{M})) \times 100 \quad (7)$$

$$\text{XylR or AraR (\%)} = 0.88 \times ((\text{C}_{\text{sugar}} \times \text{V})/(\text{xylan or arabinan content} \times \text{M})) \times 100 \quad (8)$$

where 0.90 and 0.88 are the stoichiometric factors for the conversion of glucose to equivalent cellulose, and xylose/arabinose to equivalent xylan/arabinan, respectively.

The furfural, 5-hydroxymethylfurfural (HMF), and acetic acid contents in the hydrolysates were also determined by HPLC, using an Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) ion exclusion column, operated at 30 °C, equipped with a UV–Vis detector. The mobile phase was 10 mM H_2SO_4 and the flow rate 0.6 mL/min. Acetic acid detection was performed at 210 nm, and furfural and HMF were detected at 280 nm.

The total phenolics present in the raw hydrolysates was determined as described by Mussatto et al. [43]. Briefly, the pH of the hydrolysates was raised to pH 12 by an addition of NaOH (6 mol/L), and the optical density was measured at 280 nm in a UV–Vis spectrophotometer. The total phenolics concentration (g/L), TP, was given by the Equations (9) and (10):

$$TP = 4.187 \times 10^{-2} \times ((abs_{LIG280} - abs_{PD280}) - 3.279 \times 10^{-4} \quad (9)$$

$$AbsPD280 = (C_F \times \varepsilon_F) + (C_{HMF} \times \varepsilon_{HMF}) \quad (10)$$

where Abs_{LIG280} is the absorbance at 280 nm; C_F and C_{HMF} are the concentrations (g/L) of furfural and HMF, respectively, determined by HPLC; and ε_F and ε_{HMF} are the extinction coefficients (L/g cm) of furfural (146.85) and HMF (114.00). All measurements were performed in duplicate.

2.6. Yeast Cultivation Experiments

2.6.1. Yeast Strain and Medium

All experiments were performed with *Komagataella pastoris* DSM 70877 (purchased from DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen). For inocula preparation, *K. pastoris* was cultivated in medium K, as described by Farinha et al. [44]. For the shake flasks experiments, the different hydrolysates were used as cultivation media, after being filter-sterilized (0.2 μm) and supplemented with ammonium sulfate (13.55 g/L).

2.6.2. Inocula Preparation

The inocula were prepared in 500 mL baffled shake flasks by inoculating the cryopreserved culture (1 mL) in 150 mL medium K supplemented with glucose (20 g/L) and pH adjustment to 5.0 by an addition of ammonium hydroxide (25%, *v*/*v*), followed by incubation in an orbital shaker, at 30 °C and 200 rpm, until an optical density of 2.5–3.0 (measured at 600 nm) was reached (around 40 h of cultivation).

2.6.3. Shake Flask Experiments

The yeast cultivation experiments were performed in 250 mL baffled shake flasks with 80 mL of raw hydrolysate/BSG detoxified hydrolysate, supplemented with ammonium sulfate (10 mL, 13.6 g/L) and a 10% (*v*/*v*) inoculum prepared as described above. Cultivation was performed in an orbital shaker, at 30 °C and 200 rpm, for 120–168 h. Samples (5 mL) were collected daily for cell growth and pH monitorization. At the end of the experiments, the cultivation broth was used for quantification of biomass, sugars, alcohols, and organic acids. All assays were performed in duplicate.

2.6.4. Analytical Techniques

Culture broth samples were centrifuged at 13,131× *g*, for 20 min at 4 °C, in order to separate yeast cells. The cell pellet was used to gravimetrically determine the cell dry weight (CDW), after being washed twice with deionized water and lyophilized (−110 °C, 48 h). The cell-free supernatant was used for the quantification of sugars, furfural, HMF, and acetic acid by HPLC, as described above. Xylitol concentration in the cell-free supernatant was also quantified by HPLC, with a CarboPac MA1 column (Dionex), equipped with an electrochemical detector (ED). The analysis was performed at 25 °C using NaOH (612 mM)

as the eluent, at a flow rate of 0.4 mL/min. Xylitol (Sigma-Aldrich, Darmstadt, Germany) standard solutions were used in the concentration range of 5–100 ppm.

The yields of xylitol ($Y_{xyl/S}$) and arabitol ($Y_{arab/S}$) on a substrate basis were calculated using the following equations:

$$Y_{xyl/S} = \Delta_{xylitol} / \Delta_{xylose} \quad (11)$$

$$Y_{arab/S} = \Delta_{arabitol} / \Delta_{arabinose} \quad (12)$$

where $\Delta_{xylitol}$ and $\Delta_{arabitol}$ (g/L) are the concentrations of xylitol and arabitol produced, respectively, and Δ_{xylose} and $\Delta_{arabinose}$ are the concentrations of xylose and arabinose consumed, respectively.

2.7. Statistical Analysis

All experiments were performed at least in duplicate, and results were expressed as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Characterization of the Raw Materials

The composition of the lignocellulosic materials (Figure 1) in terms of moisture, ash content, hemicellulose (xylan and arabinan), cellulose, lignin (ASL and AIL), and protein is presented in Table 2.

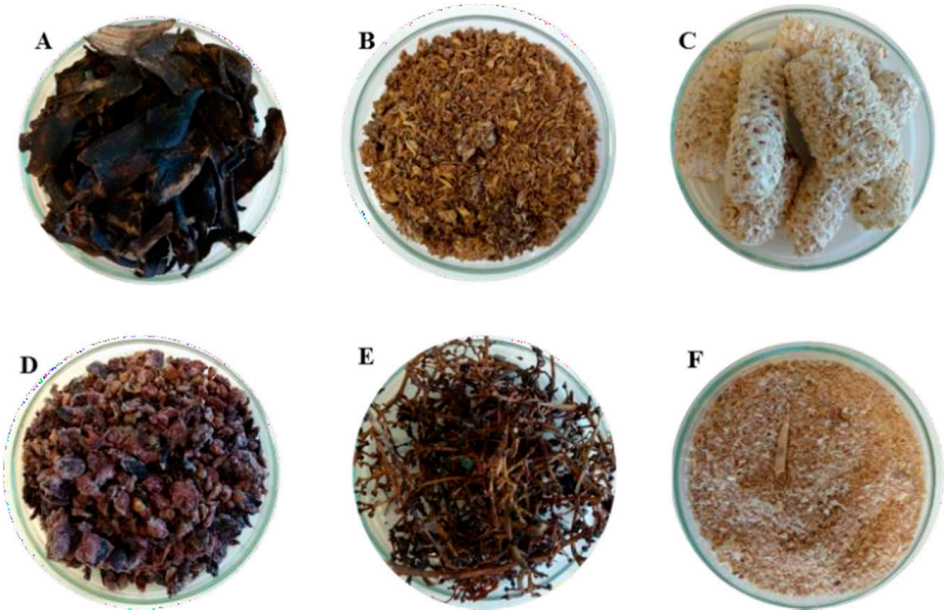

Figure 1. Photographs of the lignocellulosic materials: (**A**) banana peels, (**B**) brewer's spent grain (BSG), (**C**) corncobs, (**D**) grape pomace, (**E**) grape stalks, and (**F**) sawdust.

As shown in Table 2, the materials' moisture content was highly variable, according to their origin and nature. The highest contents were found for banana peels (89.7 ± 0.4%), BSG (72.1 ± 0.8%), and grape pomace (58.9 ± 0.6%). The remaining materials had moisture contents below 10%.

Table 2. Composition of the lignocellulosic materials (ASL, acid soluble lignin and AIL, acid insoluble lignin).

Composition	Banana Peels	BSG	Corncobs	Grape Pomace	Grape Stalks	Sawdust
Moisture (%)	89.7 ± 0.4	72.1 ± 0.8	8.9 ± 0.9	58.9 ± 0.6	9.7 ± 0.4	9.1 ± 0.5
Ash content (%) [a]	13.4 ± 1.7	1.3 ± 0.5	4.1 ± 0.5	8.7 ± 0.8	5.4 ± 0.7	0.5 ± 1.1
Cellulose (%) [a]	21.1 ± 1.3	19.0 ± 1.7	28.4 ± 0.5	12.2 ± 0.4	17.6 ± 2.2	42.8 ± 1.1
Hemicellulose (%) [a]	7.3 ± 0.5	17.6 ± 1.2	34.1 ± 1.2	7.3 ± 0.4	7.8 ± 1.0	18.0 ± 0.6
Xylans (%) [a]	4.8 ± 0.4	10.7 ± 1.0	30.2 ± 0.9	5.9 ± 0.4	3.9 ± 0.5	17.5 ± 0.6
Arabinans (%) [a]	2.5 ± 0.3	6.9 ± 0.7	3.9 ± 0.8	1.4 ± 0.2	3.9 ± 0.9	0.5 ± 0.1
Total lignin (%) [a]	14.1 ± 0.5	16.3 ± 0.6	17.1 ± 2.4	18.1 ± 0.7	18.6 ± 0.9	17.9 ± 0.5
ASL (%) [a]	6.8 ± 0.5	11.3 ± 0.2	6.3 ± 2.3	3.9 ± 0.7	7.1 ± 1.3	9.6 ± 0.6
AIL (%) [a]	7.3 ± 0.1	5.0 ± 0.2	10.8 ± 2.4	14.2 ± 0.2	11.5 ± 0.8	8.3 ± 0.7
Protein (%) [a]	6.6 ± 0.3	27.5 ± 0.5	3.4 ± 0.4	9.8 ± 0.6	9.2 ± 0.2	0.5 ± 0.1

[a]—on a dry weight basis

As expected, lignocellulosic polymers (cellulose, hemicellulose, lignin) represented a large percentage of the total dry weight of the raw materials (Table 2). Corncobs had the highest content of lignocellulosic polymers (79.6%) followed by sawdust (78.7%). BSG, grape stalks, banana peels, and grape pomace exhibited lower contents (52.9%, 44.0%, 42.5%, and 37.6%, respectively). The remaining fraction included other components such proteins, pectin, ash, fats, and oils [21].

Hemicellulose, the most relevant component for xylitol production, was present at higher contents in corncobs (34.1%), sawdust (18.0%), and BSG (17.6%). On the other hand, banana peels, grape pomace, and grape stalks contained only 7.3–7.8% hemicellulose (Table 2). Cellulose was the major component of lignocellulosic polymers in sawdust (42.8 ± 1.1%), but for the other tested raw materials it represented considerably lower of their dry weight (12.2–28.4%) (Table 2). Lignin content was similar for all the raw materials, representing 14.1% to 18.6% of the raw materials' dry weight. These values are in accordance with those reported for the same type of lignocellulosic biomass (Table 2).

Other components found in the lignocellulosic materials included proteins and ash. Low ash contents (<10%) were found for all lignocellulosic materials, except for banana peels, which had a content of 13.4 ± 1.7%. BSG had the highest protein content (27.5 ± 0.5%), while the other lignocellulosic materials had considerably lower contents (<10%) (Table 2).

The particle size distribution of BSG, grape stalks, grape pomace, and sawdust is represented in Figure 2. Corncobs and banana peels were excluded from this analysis due to their physical characteristics, since corncobs were hard to mill and banana peels were too soft, needing to be reduced to puree. As shown in Figure 2, BSG and sawdust were mainly composed of particles smaller than 1.0 mm (90.4 and 85.0 wt%, respectively). Grape stalks, on the other hand, mostly contained particles larger than 2.36 mm (69.8 wt%). In grape pomace, approximately 63.7 wt% of the particles' size was in the range of 1.0–2.36 mm (Figure 2).

Smaller particle sizes improved hydrolysis efficacy by increasing the surface area to volume ratio. Barakat et al. [45] reported dry fractionation of lignocellulose as an important step and concluded that lignocellulosic materials must have particle sizes between 0.5 and 2 mm to reach a well-accepted level of digestibility. Thus, particle size distribution of BSG, grape pomace, and sawdust are within the values reported, meaning that the effectiveness of dilute acid hydrolysis is increased.

3.2. Characterization of the Raw Hydrolysates

3.2.1. Sugar Composition and Hydrolysis Efficiency

The sugar composition of the raw hydrolysates obtained by the dilute acid hydrolysis of the lignocellulosic materials is shown in Figure 3.

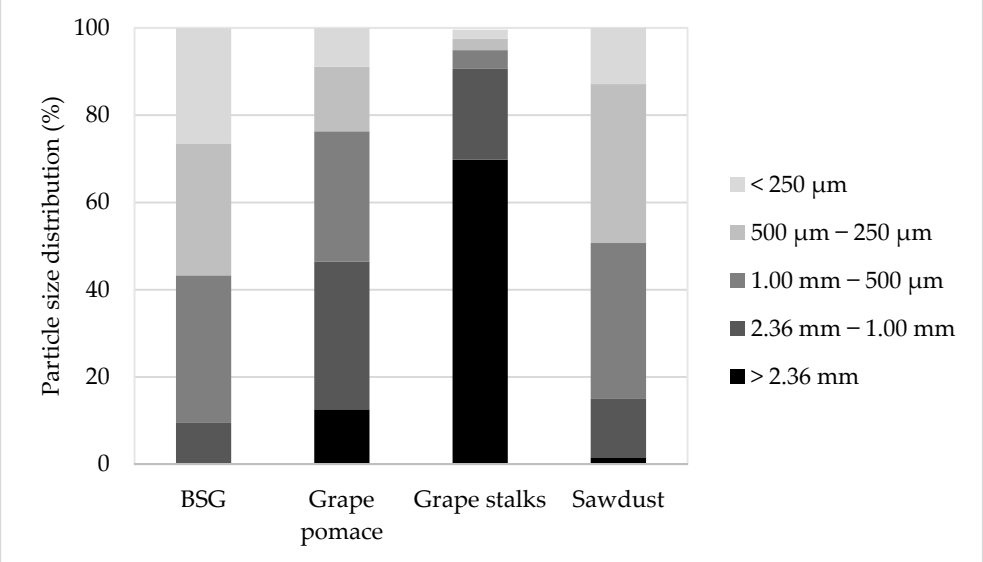

Figure 2. Particle size distribution of brewer's spent grain (BSG), grape pomace, grape stalks, and sawdust.

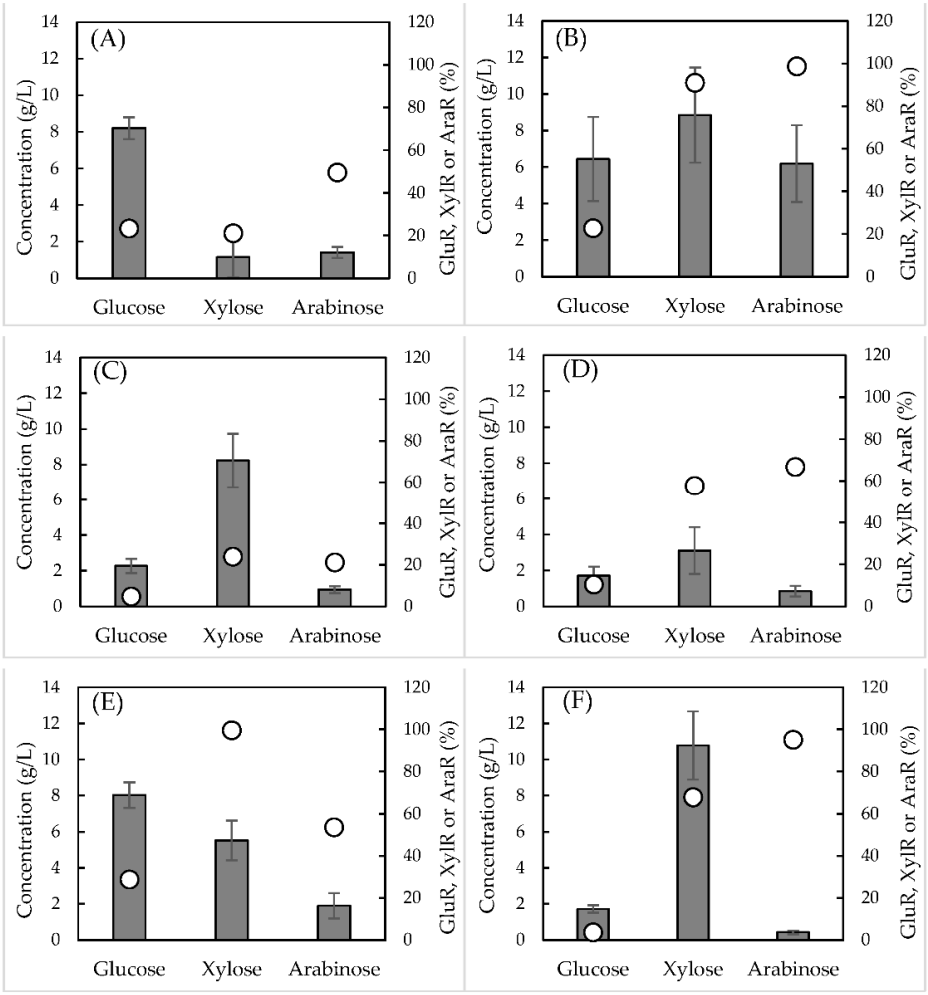

Figure 3. Sugars concentrations (■) and hydrolysis efficiencies (○) of the different raw materials: (**A**) banana peels, (**B**) brewer's spent grain (BSG), (**C**) corncobs, (**D**) grape pomace, (**E**) grape stalks, and (**F**) sawdust. GluR, XylR, and AraR represents the percentage of cellulose, xylan, and arabinan converted into glucose, xylose, and arabinose, respectively.

The highest total monosaccharides concentration was found for BSG hydrolysate (21.5 g/L) with similar contents of glucose and arabinose (6.4 ± 2.3 and 6.2 ± 2.1 g/L, respectively) and slightly higher xylose content (8.9 ± 2.6 g/L) (Figure 3B). On the other hand, only 5.66 g/L monosaccharides were attained from grape pomace, with low concentrations of glucose, xylose, and arabinose (1.7 ± 0.5, 3.1 ± 1.3, and 0.9 ± 0.3 g/L, respectively) (Figure 3D).

Higher glucose concentrations were achieved from banana peels and grape stalks (8.2 ± 0.6 and 8.0 ± 0.7 g/L, respectively) (Figure 3A,E), while corncobs and sawdust hydrolysates contained the highest concentrations of xylose (8.2 ± 1.5 and 10.8 ± 1.9 g/L, respectively), which is consistent with the high content of xylans present in these raw materials (Table 2). Moreover, apart from BSG hydrolysate, all the hydrolysates had low contents in arabinose (0.4–1.9 g/L) (Figure 3).

Compared with the literature, the xylose concentration obtained in the different raw hydrolysates was lower than the reported values. For example, Rivera and coworkers [38] reported that 7.2 g/L of xylose was obtained by hydrolysis of grape pomace under similar hydrolysis conditions but with a higher hydrolysis time (125 min). Furthermore, corncob hydrolysate's content in xylose (8.2 ± 1.5 g/L) was considerably lower than the values reported in several studies (21.7–31.2 g/L) [17,46,47], which is probably related to the fact that no mill treatment was applied to corncobs due to their hard structure.

Given the mild conditions employed for hydrolyzing the lignocellulosic materials (dilute acid), the hydrolysis efficiency of cellulose (GluR) was below 30% for all materials (Figure 3). This result is explained by the high crystallinity and polymerization degree of cellulose that requires harsher conditions for an efficient hydrolysis [48]. In contrast, the hydrolysis of hemicellulose was considerably more efficient for most materials. The highest hemicellulose hydrolysis efficiency was reached for BSG, where the xylan and arabinan fractions were almost completely converted into xylose and arabinose, respectively, with XylR and AraR values of 91.0 ± 1.8% and 98.7 ± 2.5% (Figure 3B). These values are similar to the ones reported by Carvalheiro et al. [49], who demonstrated the recovery of 95% of the BSG's xylan as xylose and 96% of the arabinan as arabinose, under similar hydrolysis conditions.

High efficiency was also achieved for the hydrolysis of xylan's fraction of grape stalks (99.6 ± 0.8%) and sawdust (67.8 ± 0.3%) (Figure 3E,F). Similar results (XylR of 89.9%) were reported by Rafiqul et al. [41]. For the remaining lignocellulosic materials, i.e., banana peels, corn cobs, and grape pomace, lower hydrolysis efficiency values were obtained for XylR (<60%) (Figure 3A,C,D).

3.2.2. Content of Hydrolysis Degradation Products

Raw hydrolysates also comprised degradation products, namely furan derivates (furfural and HMF) and aliphatic acids (acetic acid). Furfural was found in the hydrolysates of sawdust, BSG, and corncobs, while HMF was present in all hydrolysates, except grape pomace (Figure 4).

Nevertheless, the overall concentration of furan derivates was low in all the hydrolysates (<0.5 g/L). These values are in line with the reported values (0.03–0.55 g/L) and is related to the pH adjustment of the raw hydrolysates that probably induced the precipitation and instability of the compounds [39,50]. The highest HMF concentration (0.51 g/L) was found in banana peel hydrolysate, which is probably due to the high glucose content of the material that suffered degradation into furfural during the hydrolysis procedure. On the other hand, sawdust hydrolysate revealed the highest furfural concentration (0.32 g/L), resulting from the degradation of xylose (Figure 3).

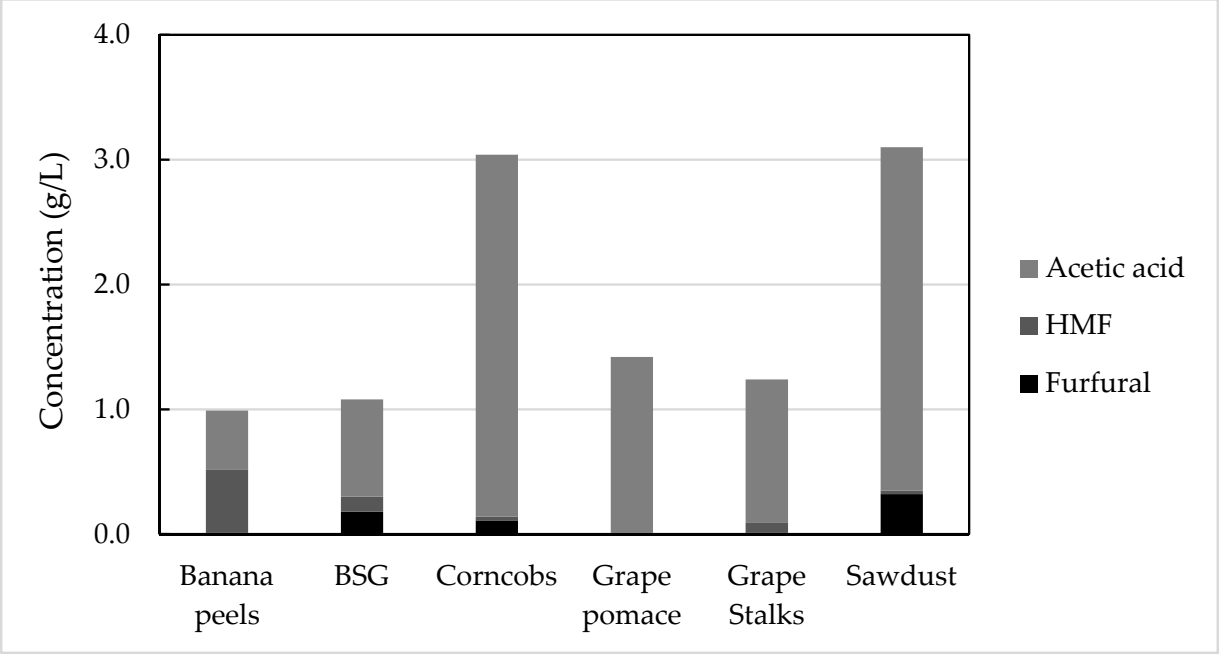

Figure 4. Concentration of furfural, HMF, and acetic acid in the raw hydrolysates.

The most relevant degradation product was the acetic acid that was present in all raw hydrolysates at concentrations ranging from 0.47 to 2.90 g/L (Figure 4). Corncobs and sawdust hydrolysates revealed the highest concentrations of acetic acid (2.90 and 2.75 g/L, respectively), which is consistent with the high xylans fraction of these raw materials (Table 2). In fact, acetic acid is derived from acetyl groups of hemicellulose, and increased concentrations of this degradation product are found in hardwoods [47,51].

3.2.3. Phenolic Compounds Content

Phenolic compounds are also present in all raw hydrolysates at concentrations between 0.19 and 1.85 g/L. Due to grape composition [52], grape pomace and grape stalk hydrolysates had the highest contents of total phenolics (1.85 and 1.62 g/L, respectively). Similarly, banana peel hydrolysate also revealed a total phenolics concentration of 1.23 g/L, which is in accordance with the reported values (1.35 g/L) [36]. Additionally to their natural origin, phenolic compounds can be obtained as a result of the lignin degradation [16]. In fact, the highest lignin fraction was found in the grape pomace and grape stalk materials, which is concomitant with the highest contents of total phenolics.

3.3. Cultivation of K. pastoris on Raw Hydrolysates

The raw hydrolysates, after pH adjustment to pH 5, were used as media for the cultivation of *K. pastoris* to evaluate their suitability to support cell growth and xylitol synthesis (Table 3). The results show that the highest biomass production was obtained for banana peels (15.18 $\pm$ 0.33 g/L) and grape stalks (14.58 $\pm$ 0.19 g/L), concomitant with the high glucose consumption (8.20 and 6.68 g/L, respectively) as observed for both assays. The obtained CDW values are slightly higher than the ones reported for the same strain (12.15 and 12.08 g/L) using glucose/xylose mixtures with similar glucose:xylose ratios (15% and 30% xylose) [33].

Table 3. Substrate consumption, cell dry weight (CDW), xylitol and arabitol production, after 120 h of cultivation.

Raw Hydrolysate	Banana Peels	BSG	Corncobs	Grape Pomace	Grape Stalks	Sawdust
Initial glucose (g/L)	8.2 ± 0.6	6.4 ± 2.3	2.3 ± 0.4	1.7 ± 0.5	8.0 ± 0.7	1.7 ± 0.2
Glucose consumed (g/L)	8.2 ± 1.5	4.9 ± 0.9	0.7 ± 0.1	1.7 ± 0.3	6.7 ± 1.2	0.1 ± 0.0
Initial xylose (g/L)	1.2 ± 1.1	8.9 ± 2.6	8.2 ± 1.5	3.1 ± 1.3	5.5 ± 1.1	10.8 ± 1.9
Xylose consumed (g/L)	1.0 ± 0.4	2.3 ± 0.9	2.2 ± 0.8	1.7 ± 0.6	3.1 ± 1.2	0.5 ± 0.2
Initial arabinose (g/L)	1.4 ± 0.3	6.2 ± 2.1	0.9 ± 0.2	0.9 ± 0.3	1.9 ± 0.7	0.4 ± 0.1
Arabinose consumed (g/L)	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.0
CDW (g/L)	15.18 ± 0.33	5.70 ± 0.94	13.70 ± 0.15	7.08 ± 0.17	14.58 ± 0.19	0.92 ± 0.09
Xylitol (g/L)	0.06 ± 0.01	1.51 ± 0.07	0.81 ± 0.05	0.36 ± 0.07	0.78 ± 0.04	0.02 ± 0.01
Arabitol (g/L)	0.02 ± 0.01	0.90 ± 0.09	0.21 ± 0.03	0.21 ± 0.02	0.34 ± 0.04	0.07 ± 0.01

Despite their low glucose contents, corncob and grape pomace hydrolysates resulted in a CDW of 13.70 ± 0.15 g/L and 7.08 ± 0.17 g/L, respectively (Table 3), which might be explained by the consumption of oligosaccharides resulting from the hydrolysis of cellulose and hemicellulose. This was not observed for sawdust hydrolysate, for which a very low glucose consumption (0.09 g/L) was noticed, possibly due to the presence of high concentrations of degradation products, especially furfural (0.32 g/L) (Figure 4), which is known to affect yeast cell growth [53].

K. pastoris was able to consume xylose from all the raw hydrolysates and converting it into xylitol, although with differing efficiencies (Table 3). Despite of the high xylose content in the sawdust hydrolysate, only traces of xylitol were detected because of the poor cell growth in the assay. The highest xylose consumption was observed in the assay performed with grape stalks (3.10 ± 1.2 g/L), but only 0.78 ± 0.04 g/L of xylitol were produced, with a $Y_{P/S}$ of 0.25 g/g on a xylose basis. For banana peel and corncob hydrolysates, even though high cell growth was attained (15.18 ± 0.33 and 13.70 ± 0.15 g/L, respectively), xylitol synthesis was low. This might be explained by the low xylose concentration available in banana peel hydrolysate (Table 3) and by the presence of high contents of inhibitory byproducts in corncob hydrolysate (Figure 4).

Despite its low biomass production (5.70 ± 0.94 g/L), BSG hydrolysate resulted in the highest production of xylitol (1.51 ± 0.07 g/L) with a xylitol yield of 0.66 g/g (Table 3). Analogous result was reported by Mussatto et al. [54] where a $Y_{P/S}$ of 0.70 g/g was achieved by the yeast *Candida guilliermondii* using BSG hydrolysate.

Arabitol was detected at low concentrations (<1.0 g/L) in the experiments carried out with BSG, corncob, grape pomace, and grape stalk hydrolysates, which is concomitant with the consumption of arabinose [55]. The highest arabitol synthesis was noticed for BSG hydrolysate (0.90 ± 0.09 g/L), which had the highest arabinose content (6.2 ± 2.1 g/L). These results revealed that *K. pastoris* can simultaneously synthesize both polyols from the same feedstock.

3.4. Optimization of K. pastoris Cultivation on BSG Hydrolysate

3.4.1. Detoxification of BSG Hydrolysate

Aiming to improve cell growth and xylitol production from BSG, several treatments were applied to the raw hydrolysate (Figure S1). As mentioned above, the pH of the initial hydrolysate (Figure S1A) was adjusted to pH 5 using $Ca(OH)_2$ (Figure S15B), which is the optimum pH for *K. pastoris* cultivation [56]. Afterwards, the raw hydrolysate was concentrated (Figure S1C) and detoxified (Figure S1D) to increase its sugars concentration and decrease the content in toxic hydrolysis degradation products, respectively. The BSG hydrolysates presented a brown coloration, with the color being intensified after the concentration treatment.

As shown in Table 4, adjusting the pH to 5 had no significant effect on the hydrolysate's composition, since only a slight reduction in the concentration of the different components was noticed, namely the loss of xylose and arabinose.

Table 4. Effect of treatments on the composition of brewers spent grain hydrolysates.

Type of Hydrolysate	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Furfural (g/L)	HMF (g/L)	Acetic Acid (g/L)
Initial	6.44 ± 1.18	8.85 ± 3.23	6.19 ± 1.78	0.18 ± 0.03	0.12 ± 0.02	0.78 ± 0.06
Raw (pH adjusted)	6.44 ± 1.19	8.13 ± 2.96	5.72 ± 1.65	0.15 ± 0.02	0.15 ± 0.02	0.76 ± 0.06
Concentrated	11.71 ± 2.16	15.22 ± 5.55	10.30 ± 2.96	0.23 ± 0.04	0.30 ± 0.05	1.14 ± 0.08
Detoxified	9.57 ± 1.76	13.00 ± 4.74	8.85 ± 2.55	0.01 ± 0.00	0.00 ± 0.00	0.72 ± 0.05

As expected, upon concentrating, the concentrations of glucose, xylose, and arabinose increased (11.71 ± 2.16, 15.22 ± 5.55, and 10.30 ± 2.96 g/L, respectively), but as a side effect, the concentration of degradation products also increased (up to 1.14 g/L) (Table 4). Therefore, to reduce the concentration of toxic compounds, a detoxification treatment using activated charcoal was applied to the concentrated hydrolysate. Activated charcoal was reported as the most effective method for the removal of furan derivates [49]. As shown in Table 4, the detoxification treatment allowed HMF to be completely removed, and furfural concentration was reduced by 96%. Nevertheless, the removal rate of acetic acid was significantly lower (37%), with the concentration obtained (0.72 ± 0.05 g/L) being similar to the value present in the raw hydrolysate (0.78 ± 0.06 g/L). In addition, the detoxification treatment also resulted in losses of sugars, which were reduced by 14–18% (Table 4). These results are comparable to those reported by Carvalheiro et al. [57], namely a high removal of furfural and HMF (92% and 68%, respectively), along with a reduction of 13% of the sugars concentrations. Moreover, the removal of acetic acid was inefficient with a removal rate of 17%.

3.4.2. Cultivation of *K. pastoris* on Detoxified BSG Hydrolysate

The concentrated BSG hydrolysate promoted biomass production (13.73 ± 0.25 g/L), but not xylitol synthesis (Table 5). In fact, the production of xylitol (1.56 ± 0.07 g/L) was similar to that obtained with the raw hydrolysate (1.51 ± 0.07 g/L), which could be explained by the presence of high concentration of degradation products, especially HMF, which could affect xylitol synthesis [58].

Table 5. Substrate consumption, cell dry weight (CDW), xylitol and arabitol production, yield of xylitol ($Y_{xyl/S}$) and yield of arabitol ($Y_{arab/S}$) on a substrate basis, after 168 h of cultivation.

Type of BSG Hydrolysate	Concentrated	Detoxified
Initial glucose (g/L)	11.90 ± 0.19	11.68 ± 2.10
Glucose consumed (g/L)	11.90 ± 0.19	11.43 ± 2.06
Initial xylose (g/L)	14.18 ± 1.04	15.65 ± 2.65
Xylose consumed (g/L)	5.89 ± 0.43	7.13 ± 1.21
Initial arabinose (g/L)	9.87 ± 0.43	9.71 ± 0.86
Arabinose consumed (g/L)	3.98 ± 0.17	1.37 ± 0.12
CDW (g/L)	13.73 ± 0.25	20.26 ± 0.25
Xylitol (g/L)	1.56 ± 0.07	3.97 ± 0.10
$Y_{xyl/S}$ ($g_{xylitol}/g_{xylose}$)	0.26 ± 0.09	0.56 ± 0.17
Arabitol (g/L)	0.43 ± 0.05	0.82 ± 0.05
$Y_{arab/S}$ ($g_{arabitol}/g_{arabinose}$)	0.11 ± 0.09	0.60 ± 0.11

The detoxification treatment, on the other hand, resulted in a significant improvement of the production of biomass and xylitol. Due to the decreased concentration of toxic compounds (Table 4), the biomass production increased to 20.26 ± 0.25 g/L, while xylitol production was improved to 3.97 ± 0.10 g/L. These results are within a range of xylitol production (1.40 and 5.31 g/L) as reported by Araújo et al. [33] for the same *K. pastoris* strain in batch bioreactor experiments using glucose (35–60 g/L) and xylose (10–35 g/L) mixtures. Analogous improvement was also reported for *Candida guilliermondii*, where the

detoxification of BSG concentrated hydrolysate revealed an increase of the xylitol/xylose yield from 0.37 to 0.55 g/g [54].

The production of arabitol was also noticed, which explains the consumption of arabinose (Table 5). As for xylitol production, the detoxification treatment applied to the concentrated hydrolysate improved arabitol production from 0.43 ± 0.05 to 0.82 ± 0.05 g/L.

Although the production of xylitol was lower than the values reported for processes based on the use of brewer's spent grain as feedstock for microbial cultivation (Table 6), the yield of xylitol on a substrate basis was within the range of those reported for *D. hansenii* CCMI 941 (0.50 g/g) or *C. guilliermondii* FTI 20037 (0.55–0.79 g/g). These results show that *K. pastoris* is a promising candidate for the development of a bioprocess for xylitol production.

Table 6. Production of xylitol by different yeast strains using several types of brewer's spent grains hydrolysates.

Microorganism	Type of Hydrolysate	Additional Treatment	Xylitol (g/L)	Y_{xyl}/s ($g_{xylitol}/g_{xylose}$)	References
K. pastoris DSM 70877	Concentrated (2×)	-	1.56 ± 0.07	0.26 ± 0.09	This study
		Activated charcoal detoxification	3.97 ± 0.10	0.56 ± 0.17	
D. hansenii CCMI 941	Concentrated (4×)	Activated charcoal detoxification	-	0.50	[57]
C. guilliermondii FTI 20037	Raw	-	-	0.65	[59]
	Raw	-	10.76	0.70	[19]
	Raw	Sugars' supplementation	62.3	0.79	[54]
	Concentrated (4×)	Activated charcoal detoxification	-	0.55	

4. Techno-Economic Analysis (TEA)

A techno-economic analysis was performed based on the literature [60] to demonstrate the feasibility of xylitol production from BSG. It is known that BSG represents around 85% of the total by-products generated by the brewing industry and is commonly disposed in landfill. Several studies [54,57,59] have reported the valorization of BSG mainly with a focus on the production of health-promoting food products such as xylitol. In fact, xylitol represents a high-value product with large profit margins, even at a low production scale, with values >5 USD·kg^{-1} and a market with a compound annual growth rate (CAGR) >6%. Techno economic evaluations revealed that BSG valorization into xylitol presented the lowest production costs (<0.35 USD·kg^{-1}) when compared to other chemicals production such as lactic acid or polyhydroxybutyrate.

The present study demonstrated that BSG has the potential to be used as a feedstock for the production of xylitol by *K. pastoris*. However, several improvements are required to make the process more attractive and economically feasible, namely optimizing the hydrolysis conditions to increase the xylose concentration in the acid hydrolysate, thereby maintaining the inhibitory compounds at low contents. In addition, the process scale-up is mandatory to investigate yield and productivity on a larger scale.

5. Conclusions

K. pastoris, a nonpathogenic GRAS yeast, demonstrated the ability to synthesize the sugar alcohol xylitol upon cultivation using sugar rich hydrolysates obtained from different lignocellulosic materials. Among the tested hydrolysates, brewer's spent grain hydrolysate resulted in the highest conversion of xylose into xylitol. Additionally, *K. pastoris* was able to synthesize arabitol based on the consumption of arabinose present in the hydrolysate, a feature that had not been reported previously. These findings show the suitability of using

K. pastoris to develop a bioprocess for the valorization of lignocellulosic materials by their conversion into value-added sugar alcohols.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11125516/s1, Figure S1: Photographs of: (A) the initial BSG hydrolysate and the BSG hydrolysate after (B) pH adjustment, (C) concentration and (D) detoxification treatments.

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