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Cardoon Hydrolysate Detoxification by Activated Carbon or Membranes System for Bioethanol Production

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Abstract: Advanced biofuels incorporation into the transportation sector, particularly cellulosic bioethanol, is crucial for attaining carbon neutrality by 2050, contributing to climate changes mitigation and wastes minimization. The world needs biofuel to be commercially available to tackle the socioeconomic challenges coming from the continued use of fossil fuels. *Cynara cardunculus* (cardoon) is a cheap lignocellulosic raw biomass that easily grows in Mediterraneous soils and is a potential renewable resource for a biorefinery. This work aimed to study the bioethanol production from cardoon hemicellulosic hydrolysates, which originated from dilute sulfuric acid hydrolysis pretreatment. A detoxification step to remove released microbial fermentative inhibitors was evaluated by using both activated carbon adsorption and a nanofiltration membrane system. The *Scheffersomyces stipitis* CBS5773 yeast and the modified *Escherichia coli* MS04 fermentation performances at different experimental conditions were compared. The promising results with *E. coli*, using detoxified cardoon by membrane nanofiltration, led to a bioethanol volumetric productivity of 0.30 $g{\cdot}L^{-1}{\cdot}h^{-1}$, with a conversion efficiency of 94.5%. Regarding the *S. stipitis*, in similar fermentation conditions, volumetric productivity of 0.091 $g{\cdot}L^{-1}{\cdot}h^{-1}$ with a conversion efficiency of 64.9% was obtained. Concluding, the production of bioethanol through detoxification of hemicellulosic cardoon hydrolysate presents a suitable alternative for the production of second-generation bioethanol, especially using the modified *E. coli*.

Keywords: bioethanol; cardoon hemicellulosic hydrolysate detoxification; activated carbon adsorption; membrane nanofiltration; microbial fermentation; *Scheffersomyces stipites*; modified *Escherichia coli*

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1. Introduction

Global energy consumption has been rising during the last decades, and the transports sector is associated with a significant contribution to fuels' burning, increasing greenhouse gas emissions (GHG). The European Union (EU) put forth renewable energies directives in 2015 and gradually raised its targets, envisaging carbon neutrality by 2050 [1]. Specific governments' regulations and policys started to increase the reduction of fossil fuels' utilization, improving jobs and the bioeconomy and simultaneously boosting the net-zero goal [2].

According to the circular economy model, investigation of renewable resources as raw materials is mandatory for feeding processes and bioprocesses for biobased fuels, chemicals

and materials. The global climate crisis raised environmental sustainability concerns by modern society, pushing for the green transition from fossil to biomass resources [3].

Bioethanol is the most produced biofuel at an industrial-scale level. However, it is mainly obtained from corn and sugarcane, competing directly with the food chain. Therefore, research has been focused on bioethanol production from lignocellulosic sources, which are the most abundant and cheapest forms of biomass. Moreover, according to the circular economy model, this approach contributes to a more efficient waste-management system. In this context, biorefining is the best contribution to bioeconomy and sustainability, lowering fossil dependence and increasing demand for second-generation biofuels for transports, as well as biobased products for other specific applications. In several European biorefineries, the conversion of residual lignocellulosic biomass (LCB) began with bioethanol production for incorporation in bioethanol–diesel blends, contributing to the drop of transports' combustion carbon footprint [4].

LCB presents a morphological complex macromolecular structure mainly constituted by the cellulose and hemicelluloses polysaccharides and the lignin macromolecule being a suitable feedstock for the production of valuable bioproducts. However, to achieve such a goal, it is necessary (1) to choose a suitable pretreatment for delignification, usually separating the majority of hemicelluloses together with the lignin; (2) to perform an efficient polysaccharides hydrolysis in order to attain the release of monosaccharides; and (3) to successfully perform their biotransformation, usually by bioprocesses [5].

Many LCB feedstocks were researched for different biofuels applications, namely for biomethane [6], biodiesel [7] and recently for aviation utilization [8].

Cynara cardunculus (cardoon) grows in Mediterraneous soils, without needing irrigation or fertilizers, being a potential feedstock crop for a biorefinery due to its different biological activities and several studied applications [9]. Espada et al. (2021) [10] made the life-cycle assessment for bioethanol production from cardoon biomass, using different process technologies. They also compared these processes with other lignocellulosic feedstocks' bioethanol processes concerning environmental behaviors. In addition, a comparative study between bioethanol and fossil gasoline was performed, indicating that the steam explosion process for bioethanol was environmentally superior, with an improved reduction in the primary energy demand (80%) and greenhouse gas emissions (45%) [10].

Research with different cardoon pretreatments for delignification was already studied, providing (1) a solid cellulosic fraction already hydrolyzed and valorized; and (2) a liquid fraction, a mixture of lignin residues and hemicelluloses' degradation monosaccharides, a hydrolysate. The solid fraction hydrolysis released monosaccharides, mainly glucose, that were successfully evaluated for bioethanol production [11]. Aligned with the biorefinery concept and the circular economy model, the second fraction, the liquid hydrolysate rich in solubilized monosaccharides, mainly xylose, should be investigated in spite of having some soluble lignin derivatives. To efficiently convert these C5 monosaccharides into bioethanol, a previous appropriate strategy should be applied for inhibitors' removal, allowing the subsequent C5 microbial fermentation [12]. However, just some microbial species, such as *Scheffersomyces stipitis*, are natural C5-sugars consumers, and due to the high availability of xylans in lignocellulosic materials, research for adapting this efficient species [13] to obtain a more efficient engineered strain has been published [14].

Microbial inhibitors commonly released during LCB delignification were chemically identified as phenolics, furan aldehydes and aliphatic acids. They were associated with different harmful microbial effects [15], requiring precise technologies to reduce their contents in order to allow successful microbial fermentations.

Several methodologies have investigated hydrolysates detoxification, such as the work proposed by Xavier et al. (2010) [16], where some deacidificant yeasts and also ion exchange for acetic deacidification of a pulp and paper subproduct reach in xylose, namely spent sulphite liquor, were evaluated. With the same liquor, Pereira et al. (2012) [5] used the filamentous fungus *Paecilomyces variotii* to perform its biodetoxification by removing acetic

acid, gallic acid and pyrogallol, using a sequential batch reactor (SBR) strategy, allowing *S. stipitis* bioethanol fermentation.

Activated carbon adsorption is widely applied for purification due to the high porosity of the material being also tested for lignocellulosic hydrolysates detoxification, namely for phenolics, furfural and hydroxymethylfurfural removal. Weissgram et al. (2015) [17] detoxified spent sulfite liquor and could decrease phenolic content, improving bioethanol fermentation. Sarawan and co-workers reported, in 2019 [18], a decrease of toxic compounds without a significant loss in fermentable sugars, providing an improvement in bioethanol fermentation. Recently, *Cistus ladanifer* hydrolysate detoxification was successfully performed by Alves-Ferreira et al. (2022) by using activated charcoal adsorption [19].

Arminda et al. (2021) [20] used olive-tree-pruning residues to produce activated charcoal and characterized its adsorption. After optimization, they reported its efficient application in detoxification of a hydrolysate coming from the same type of residues [20].

Modern industrial membrane systems are appearing and being implemented to clean effluents or recover and purify several specific compounds. Recently, pervaporation membranes were investigated and discussed for potential bioethanol recovery [21]. Membranes' systems were also studied for hydrolysates detoxification, namely for specific inhibitors removal using nanofiltration, diafiltration and pervaporation, thus preventing sugars' loss [22,23].

This work aimed to study bioethanol production from the hemicellulosic hydrolysates originated by dilute sulfuric acid–hydrolysis pretreatment of cardoon. Detoxification of hydrolysate for inhibitors removal was studied by using activated carbon adsorption or performing membrane nanofiltration. A comparison of *S. stipitis* CBS5773 yeast and modified *Escherichia coli* MS04 bacterium for the conversion of the respective detoxified hydrolysates containing xylose and glucose to second-generation bioethanol production is presented and discussed.

2. Materials and Methods

2.1. Microorganisms

The yeast *S. stipitis* CBS5773 was kindly provided by the National Laboratory of Energy and Geology. The ethanologenic bacterium *E. coli* MS04 was kindly provided and developed by the Institute of Biotechnology of the National Autonomous University of Mexico. The MS04 strain was genetically modified to tolerate acetic acid and metabolize glucose and xylose to produce bioethanol [24]. All the experimental work with the microorganisms was performed after sterilization of all materials and keeping sterile procedures.

2.2. Chemicals

Xylose, arabinose, agar from Sigma-Aldrich (St. Louis, MO, USA) and kanamycin monosulphate were purchased from Duchefa-Biochemie (Haarlem, The Netherlands); yeast extract, malt extract and Luria-Bertani Broth (LB medium) were purchased from Merck (Darmstadt, Germany); peptone was purchased from Fluka (Charlotte, NC, USA); calcium carbonate and glucose were purchased from Normapur (Radnor, PA, USA).

2.3. Raw Biomass and Pretreatment

Three-year-old dry cardoon was collected from a farm of the Agrarian School of Beja, Portugal. The biomass of stalk and leaves of cardoon was previously characterized [25], and it was size reduced and sieved to obtain particles 40–60 mesh. Then, 50 g was pretreated with diluted acid (H_2SO_4 6.7 wt%), with a ratio of 10 g acid solution/g biomass, using cardoon at 121 °C and 1 bar during 55 min in autoclave, Uniclave, AJC, Portugal. The solid fraction of cardoon was vacuum-filtered, and the supernatant, the liquid fraction, was denominated hydrolysate, and it was stored at 4 °C.

2.4. Hydrolysate Detoxification by Activated Carbon

The inhibitors adsorption in activated charcoal (Sigma C3345-500G untreated powder, 100–400 mesh) was assayed as follows: firstly, the charcoal was activated by adding NaOH 5M (ratio of 1:5 (w/v)) and stirring at 150 rpm for 1 h at 30 ± 1 °C in an incubator (TEQ, JTC, Portugal). Then 45.9 g and 53.7 g of activated carbon were added progressively to the hydrolysate (pH 1.12), until reaching pH 5.5 for *S. stipitis* and pH 7.0 for *E. coli* assays, respectively. Then 200 mL of each treated hydrolysate was maintained under stirring at room temperature for 1 h, and, finally, the activated carbon was removed by vacuum filtration, and both hydrolysates were stored at 4 °C.

2.5. Hydrolysate Detoxification by Membrane Nanofiltration

Membrane filtration was performed by a diananofiltration experiment, considering the satisfactory results previously obtained by Brás et al. (2013) [26] with a hydrolysate. The experimental setup comprised a GE-Sepa CF cross-flow module (GE Osmonics, Minnetonka, MN, USA) and a high-pressure feed pump (Hydra-cell model G13, Wanner Engineering Inc., Minneapolis, MN, USA). The effective membrane area used was 140 cm^2. According to previous studies (data not shown), the membrane selected was the NF20 (DOW, Midland, MI, USA). During the dianofiltration, the transmembrane pressure applied was 26 bar, being the permeate continuously removed, and the feed volume held with the addition of deionized water. Samples of feed and permeate were collected periodically during the experiment. The experiment was operated till a near-total removal of inhibitors (under quantification limit by high-pressure liquid chromatography (HPLC)).

2.6. Hydrolysates Concentration

The hydrolysates were concentrated two times at 50 °C and 10 rpm, using a rotary evaporator Hei-VAP series, Heidolph Instruments, Germany. This process also allowed the removal of some volatile inhibitors, such as furfural.

2.7. Microbial Fermentations

2.7.1. Solid Culture Media

For *S. sitipitis* plate growth and maintenance, the synthetic solid culture medium, Yeast Medium of Peptone (YMP), was used. It was prepared in distilled water with yeast extract, 3.0 g/L; malt extract, 3.0 g/L; peptone, 5.0 g/L; xylose, 10 g/L; and agar, 15 g/L.

For *E. coli* MS04 plate growth, the synthetic solid culture medium, Luria-Bertani Broth (LB), (tryptone 10.0 g/L, yeast extract 5.0 g/L and NaCl 10.0 g/L) was used. The LB maintenance medium was supplemented and prepared for Petri dishes: LB, 20.0 g/L; xylose, 20.0 g/L; kanamycin monosulfate, 30.0 mg/L; and agar, 15.0 g/L in distilled water.

2.7.2. Liquid Culture Media

For *S. sitipitis* fermentations, YMP was prepared by using double sugar concentration of the detoxified hydrolysate, with activated charcoal and the pH corrected to 5.5. The composition was as follows: yeast extract, 3.0 g/L; malt extract, 3.0 g/L; peptone, 5.0 g/L; xylose, 27.5 g/L; and glucose, 6.9 g/L.

For *E. coli* MS04 fermentations, the LB liquid medium was prepared to equalize the sugars concentrations of the detoxified hydrolysate with activated carbon. Calcium carbonate was added to stabilize the pH 7.0 along fermentation for all experiments. The LB broth composition was as follows: 20 g/L; calcium carbonate, 50 g/L; xylose, 13.4 g/L; arabinose, 0.3 g/L; and glucose, 3.3 g/L.

2.7.3. Pre-Inoculum and Inoculum Preparation

Pre-inoculums were prepared by transferring one colony from each *E. coli* MS04 or *S. stipitis* Petri dish to 250 mL Erlenmeyer flasks with 100 mL of the respective liquid medium. The *S. stipitis* was incubated at 30 ± 0.5 °C and 150 rpm, and the *E. coli* MS04 was incubated at 37.0 ± 0.5 °C and 150 rpm in the orbital incubator. After 12 h, the inoculums

were centrifuged at 5000× *g* (Hermle Labortechnik Z 323K, Wehingen, Germany), the supernatant was discharged and the cells were resuspended in NaCl 0.9% (*w*/*v*). After second centrifugation at 5000× *g*, the cells were resuspended in 5 mL of each respective liquid medium for obtaining an initial optical density between 0.200 and 0.300 in the fermentation assays.

2.7.4. Fermentation Assays

S. stipitis fermentations were carried out in duplicates in 250 mL Erlenmeyer flasks with a working volume of 50 mL and incubated at 30 ± 0.5 °C and 150 rpm:

(i) Untreated hydrolysate;
(ii) Synthetic culture medium YMP, pH 5.5, with the same sugar concentration as the detoxified hydrolysate;
(iii) Hydrolysate detoxified by activated carbon and concentrated, pH 5.5;
(iv) Hydrolysate detoxified by membranes and concentrated, pH 5.5.

E. coli MS04 fermentations were carried out in duplicates in 250 mL Erlenmeyer flasks with a working volume of 45 mL and incubated at 37.0 ± 0.5 °C and 150 rpm:

(i) Untreated hydrolysate;
(ii) Synthetic LB medium, pH 7.0, with the same sugar concentration as the detoxified hydrolysate;
(iii) Hydrolysate detoxified by activated carbon and concentrated, pH 7.0;
(iv) Hydrolysate detoxified by membranes and concentrated, pH 7.0.

Samples were collected over time to monitor the pH, biomass at 600 nm for *S. stipitis* and 550 nm for *E. coli*. Then, after respective centrifugation sugars, aliphatic acids, bioethanol, 5-hydroxymethylfurfural (HMF) and furfural were quantified by HPLC.

2.8. HPLC Analysis

Quantitative analysis of D-glucose, D-xylose, L-arabinose, acetic acid, formic acid, levulinic acid, lactic acid, 5-hydroxymethylfurfural (HMF), furfural and bioethanol present in the hydrolysates was performed by using an HPLC system (Merck LaChrome Hitachi, Tokyo, Japan), pumping a flow rate of 0.6 mL min^{-1} of H_2SO_4 (0.005M) as mobile phase through a cation exchange column 300 × 7.8 mm Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) at 50 °C. The quantification of sugars, aliphatic acids and bioethanol was performed by using the L-7490 refractive index (IR) detector, while the quantification of HMF and furfural was made by using the UV–VIS detector L-7420.

2.9. Kinetic and Stoichiometric Parameters Calculation

The biomass concentration was determined by dry biomass per unit of absorbance, as described by Vargas-Tal et al. [27].

The ethanol yield, $Y_{P/S}$, per substrate (glucose and xylose) (g/g) was calculated by using Equation (1):

$$Y_{P/S} = \frac{[P]_{max} - [P]_i}{[S]_i - [S]_{max}} \quad (1)$$

where P_i and S_i are the initial product and substrate concentrations, and P_{max} and S_{max} are the maximum bioethanol concentration attained and the respective substrate concentration.

Since the theoretical yield for ethanol is 0.51 g/g, the conversion efficiency (*CE*) was determined according to Equation (2):

$$CE\ (\%) = \frac{Y_{P/S}}{0.51} \times 100 \quad (2)$$

Finally, the maximum ethanol productivity (P_{Etmax}) (g/L·h) determined at the time corresponding to the maximum bioethanol concentration, P_{max} (g/L), obtained in the fermentation, was calculated by Equation (3):

$$P_{Etmax} = \frac{[P]_{max}}{t_{max}} \quad (3)$$

where t_{max} (h) is the time corresponding to the maximum ethanol concentration, P_{max}.

2.10. Statistical Analysis

For the data subjected to analysis of variance [28], the means for each trait were separated by Fisher's least significance difference test, applying a threshold of 0.05. Values recorded as percentages were subjected to angular transformation prior to the analysis of variance; untransformed data are reported and discussed.

3. Results and Discussion

3.1. Effect of Detoxification Processes on the Composition of Cardoon Hydrolysates

3.1.1. Hydrolysate Composition

The goal of pretreating cardoon by acid hydrolysis was to promote the lignocellulosic biomass delignification by separating the solid fraction, mainly composed of cellulose, from the liquid fraction. This last one, resulting from the lignin removal, contains the lignin derivatives and the hydrolyzed hemicelluloses that became soluble monosaccharides. The separated solid fraction was the object of another previous work on the evaluation of potential for bioethanol [25], and the remaining liquid fraction (after vacuum filtration) was called the hydrolysate, which is the object of the work here presented.

The HPLC analysis of this hydrolysate evaluated the availability of fermentable sugars and revealed the presence of compounds derived from lignin, already described as traditional lignocellulosic microbial inhibitors, namely aliphatic acids and furan aldehydes [15]. Table 1 presents the composition of this cardoon hydrolysate, which is referred to as untreated hydrolysate. The most concentrated sugar was xylose, at 11.48 g/L; then glucose, at 2.57 g/L; and finally arabinose, at 1.02 g/L. As expected, the most abundant inhibitors present in the hydrolysate were acetic acid and furfural, as previously reported by Zhang et al. (2015) [29]. The concentrations of acetic acid and furfural were 3.82 and 4.88 g/L, respectively.

Table 1. Cardoon hydrolysates' composition on monosaccharides and inhibitors before and after activated carbon detoxification and concentration.

Hydrolysates Detoxified by Activated Carbon	Hydrolysates' Composition (g/L)							
	Glu [1]	Xyl	Ara	LV	AA	FA	Furfural	HMF
Untreated hydrolysate	2.57	11.48	1.02	0.26	4.48	3.32	2.19	0.04
Detoxified at pH 5.5	2.36	9.68	0.47	0.22	3.27	2.71	0.05	0.01
Detoxified at pH 7.0	2.29	9.28	0.53	0.20	3.92	3.25	0.00	0.00
Detoxified at pH 5.5 and concentrated	3.44	13.71	0.85	0.28	4.50	3.93	0.00	0.01
Detoxified at pH 7.0 and concentrated	3.27	13.37	0.75	0.56	5.79	4.79	0.00	0.01

[1] Glu, glucose; Xyl, xylose; Ara, arabinose; LV, levulinic acid; AA, acetic acid; FA, formic acid; HMF, hydroxymethylfurfural.

The xylose was the most concentrated sugar in this hydrolysate, as expected, since it is the major monosaccharide present in hemicelluloses, namely in xylans. In a similar work by Ballesteros et al. (2008) [30], a maximum yield of xylose of 12.4 g per 100 g of cardoon was achieved after hydrolysis with sulfuric acid at 0.2% (*w*/*w*), at 180 °C for 10% (*w*/*v*) of solid

concentration. In the present study, a lower yield of xylose of 8.11 g per 100 g of cardoon was obtained at a lower temperature, 121 °C, using a higher concentration of sulfuric acid, 6.7% (*w*/*w*). This yield decreased, since some of the obtained xylose was degraded to furfural and formic acid, which had concentrations of 2.19 and 3.32 g/L, respectively (Table 1). This type of degradation increasing inhibitors content was previously described by Pamqvist et al. (2000) [31].

3.1.2. Detoxification of Cardoon Hydrolysate by Activated Carbon Adsorption

To avoid inhibitor effects from different compounds in microbial fermentations, adsorption on activated carbons was attempted to eliminate or decrease such compounds. The hydrolysate was detoxified with activated carbon at pH 5.5, the proper pH for *S. stipitis* fermentation, and also at pH 7.0, the adequate pH for *E. coli*. After that, a following step of evaporation was performed for regenerating sugars' concentration, since a concomitant loss of sugars was registered, decreasing their concentration. The results of both steps are presented in Table 1**,** and they clearly show that the inhibitor compounds furfural and HMF were adsorbed on the activated carbons, revealing the treatment effectivity for the furan aldehydes. On the other hand, the adsorption of organic acids was low, and also sugars adsorption was registered. The best composition of the hydrolysate was detected for the pH 5.5 detoxification assay, followed by evaporation, since the organic acids content was lower. Moreover, sugars loss was slightly lower, as xylose, glucose and arabinose had higher final concentrations, i.e., 13.37, 3.27 and 0.75 g/L, respectively. This treatment eliminated the furanic aldehydes but was not fully effective, since the removal of the aliphatic acids was not complete; they were not considerably adsorbed in the activated carbon remaining the concentrations of acetic and formic acids significant. In a similar work with wood instead of cardoon, the detoxification of hydrolysates with activated carbon adsorption was also found to be promisor before the fermentation by the yeast *Debaryomyces hansenii* [32].

3.1.3. Detoxification of Cardoon Hydrolysate by Membrane Nanofiltration

Hydrolysate detoxification was also performed by nanofiltration, on diananofiltration mode. Table 2 shows the results of hydrolysate detoxification by membrane nanofiltration. As observed, concerning fermentation inhibitors' removal, the process was very efficient, eliminating all the toxic compounds entirely or almost completely (85, 99, 100, 95 and 99% for levulinic acid, acetic acid, formic acid, furfural and hydroxymethylfurfural, respectively). On the other hand, the use of this membrane resulted in a 31% xylose loss (from 11.81 to 8.13 g/L). With the selected membrane presenting a molecular weight cut-off of 400 Da [26], a rejection of 70% for xylose (MW 150, 13 g/mol) was obtained, thus explaining the value for xylose depletion. After detoxification, a concentration step was performed in order to increase sugars' concentration and decrease the feed volume, with final values presented on Table 2.

Table 2. Cardoon hydrolysates' composition on monosaccharides and inhibitors before and after nanofiltration membrane detoxification and concentration.

Hydrolysates Detoxified by Membrane Nanofiltration	Hydrolysates' Composition (g/L)							
	Glu [1]	Xyl	Ara	LV	AA	FA	Furfural	HMF
Untreated hydrolysate	2.47	11.81	0.59	0.26	3.73	0.386	2.02	1.411
Detoxified by membrane	2.47	8.13	0.59	0.04	0.04	0.00	0.10	0.01
Detoxified by membrane and concentrated	3.43	11.24	0.81	0.05	0.06	0.00	0.00	0.01

[1] Glu, glucose; Xyl, xylose; Ara, arabinose; LV, levulinic acid; AA, acetic acid; FA, formic acid; HMF, hydroxymethylfurfural.

Compared to the activated carbon-adsorption methodology, the removal of fermentation inhibitors with membrane nanofiltration was fully effective: formic acid, furfural

and HMF were almost entirely eliminated, and only insignificant residues of levulinic and acetic acids, 0.05 and 0.06 g/L, respectively, were detected. Concerning sugars, after the concentration step, the hydrolysates' composition was similar for both methodologies, membrane and activated carbon (Tables 1 and 2). Concluding, the treatment by membrane technology was the most promising method for detoxifying cardoon hydrolysate prior to fermentations.

3.2. Bioethanol Production by S. stipitis Fermentation

Hydrolysates of cardoon were used to the fermentative bioethanol production by *S. stipitis*. For this purpose, four different sets of fermentation media were evaluated: (i) synthetic medium, (ii) non-detoxified cardoon hydrolysate, (iii) hydrolysate treated with active charcoal and (iv) hydrolysate filtered with membrane. The process efficiency was compared in terms of maximum bioethanol concentration, yield and productivity. For both untreated hydrolysates and activated-carbon-treated hydrolysate, no bioethanol production was detected. The untreated cardoon hydrolysate contains a high concentration of inhibitory compounds, namely furfural and acetic acid, which were initially found at concentrations of 2.19 and 4.48 g/L, respectively. Since *S. stipitis* is intolerant to furfural [33] and acetic acid [24], its metabolism was completely inhibited. This yeast requires a detoxification pretreatment of cardoon hydrolysates to remove inhibitory compounds. Indeed, after treatment with activated carbon, even with complete elimination of furfural, the acetic acid concentration was still 4.50 g/L, and the hydrolysate led to the same behavior. The negative effect of acetic acid concentration in fermentations carried out by *Pichia stipitis* (now called *S. stipitis*) was previously verified in the study of Xavier et al. (2010) [16]. In the presence of 9.5 g/L of acetic acid, the yeast was inhibited entirely, neither consuming monosaccharides nor producing bioethanol [16]. In this work, with a concentration of 4.50 g/L of acetic acid, despite some sugar consumption (7.05%), the bioethanol production was also not detected, thus evidencing the need for an effective detoxification process of inhibitors removal, a condition that was not found in the detoxification process carried out with this activated carbon-adsorption experiment. As also reported by other authors, activated carbon could not remove high amounts of acetic acid [34].

Since activated carbon was not an effective pretreatment, the hydrolysate solution was processed with nanofiltration membrane, a more selective and efficient pretreatment for the removal of inhibitors.

The results of monosaccharides, glucose (C6) and xylose (C5), bioethanol and biomass concentrations during the fermentation of the hydrolysate detoxified by membrane nanofiltration are depicted in Figure 1. The arabinose, a C5 sugar, is not represented in the figures, since a very low concentration remains almost constant during all the experiments. The pH remained nearly constant at 5.5.

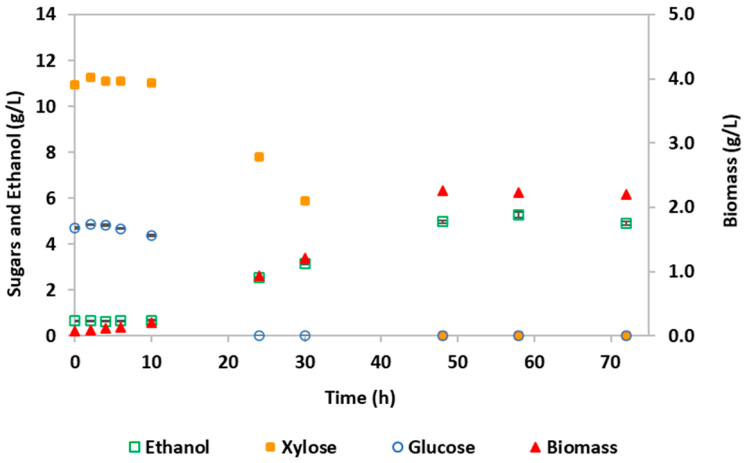

Figure 1. Time course of *S. stipitis* fermentation using the hydrolysate detoxified by membrane nanofiltration: glucose, xylose, bioethanol and biomass concentrations (50 mL, 150 rpm, initial pH 5.5 and 30 °C).

Glucose consumption started after the 6 h of lag phase, and, certainly, its total consumption was before 24 h, the time for exhaustion detection. According to diauxic behavior already described for xylose-fermenting yeasts [35], just after glucose exhaustion, *S. stipitis* should have started the xylose consumption by the pentose-consuming metabolism, with its complete exhaustion registered at 58 h. Regarding bioethanol production, the maximum concentration of 6.58 g/L was obtained at 58 h of fermentation. Some low concentrations of sugar dimers or even arabinose should be the substrates for the slight increase in bioethanol concentration. Nevertheless, at the end of the assay, a slightly decreased concentration of bioethanol of 4.90 g/L was found, indicating a low bioethanol reassimilation for *S. stipitis* maintenance.

Fermentation of the synthetic YMP medium was performed for comparison purposes (control fermentation). The profile of sugar consumption, cell growth and bioethanol production by *S. stipitis* is presented in Figure 2. It started glucose consumption very fast, as there was no lag phase. In fact, all the inoculums were prepared with the synthetic media, and this is the reason why an adaptation phase was not detected. Glucose was consumed faster, and when glucose was exhausted, after 8 h of fermentation, the consumption of xylose was started, which finished entirely after 20 h. Overall, 15.6 g of glucose and xylose sugars was consumed, leading to a bioethanol production of 6.58 g/L. This production was 1.25 times higher than that with the cardoon hydrolysate treated by membrane. Additionally, no lag phase was shown when compared to the fermentation using the hydrolysate pretreated with membrane, since the inoculum was made with the same medium (all the fermentation inocula were made with synthetic media). This medium, without any inhibitory compound, determined the very fast adaptation of *S. stipitis*.

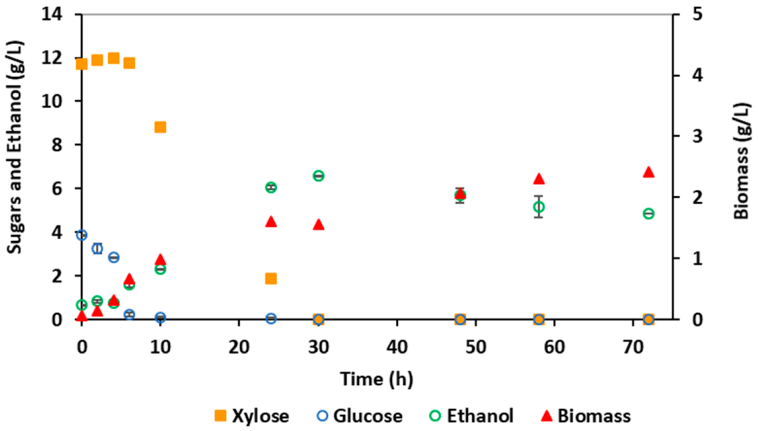

Figure 2. Time course of *S. stipitis* fermentation using the YPM synthetic medium, glucose, xylose, bioethanol and biomass concentrations (50 mL, 150 rpm, initial pH 5.5 and 30 °C).

The kinetic and stoichiometric parameters obtained in *S. stipitis* fermentations using the hydrolysate detoxified with membrane and the synthetic YMP medium are presented in Table 3. Fermentation performed with synthetic YPM medium produced better results than those obtained with hydrolyzate detoxified with membrane. This is particularly evident when looking at the maximum bioethanol concentration (6.58 vs. 5.28 g/L) and at the maximum bioethanol productivity (0.22 vs. 0.091 g/L·h), the latter depending on the former. For both of these parameters, the difference was found to be statistically significant. The yield in ethanol obtained with synthetic YPM medium (0.42 g/g) was higher than that obtained with the hydrolyzate detoxified with membrane (0.33 g/g); the latter data are reflected in the corresponding conversion coefficients (82.9% vs. 64.9%). As mentioned above, the time to attain the maximum ethanol concentration should be reduced by decreasing the lag phase, which could be performed by preparing the inoculum in the same medium as the fermentation. However, the bioethanol production by ligninolytic hydrolysates would always be lower than by synthetic media with pure sugars addition; the multiple constituents, even in low concentrations, can promote any inhibitory effects on the fermentative metabolic pathways.

Table 3. Kinetic and stoichiometric parameters obtained in the fermentations by *S. stipitis* using the synthetic YMP medium and the hydrolysate detoxified by membrane.

Parameters [1]	Hydrolysate Detoxified by Membrane Nanofiltration	Synthetic YMP Medium	Significance [2]
$Y_{P/S}$ (g/g)	0.33	0.42	NS
Et_{max} (g/L)	5.28 (58 h)	6.58 (30 h)	**
CE (%)	64.9	82.9	NS
P_{Etmax} (g/L·h)	0.091	0.22	***

[1] $Y_{P/S}$, ethanol yield; Et_{max}, maximum bioethanol concentration; CE, conversion efficiency; P_{Etmax}, maximum ethanol productivity. [2] NS, not significant; ** and *** significant at $p \leq 0.01$ and $p \leq 0.001$, respectively.

3.3. Bioethanol Production by E. coli MS04

In order to improve bioethanol production, another microorganism genetically modified was evaluated, namely *E. coli* MS04, since it is more tolerant to acetic acid and it is able to metabolize both glucose and xylose to bioethanol. The fermentations were carried out with the hydrolysates detoxified with activated carbon and membrane nanofiltration with an initial media pH of 7. A control fermentation using the synthetic medium LB was also performed.

As the previous fermentations by *S. stipititis*, the hydrolysate treated with activated carbon did not lead to any bioethanol production. Fernández-Sandoval et al. [24] reported a linear improvement in this strain growth and also in bioethanol production with increasing

concentration of acetic acid at a pH of 7. However, in this work, performed in Erlenmeyer flasks with an initial pH of 7, the addition of calcium carbonate led to a pH increase to higher values, attaining 8.2 at the end of the assay, which affected the performance of *E. coli* MS04.

On the other hand, bioethanol could be produced in the fermentation carried out with the hydrolysate detoxified by membrane nanofiltration. The results depicted in Figure 3 show a lag phase of nearly 3 h, followed by glucose consumption being the glucose depletion registered at 10 h. At the same time, xylose already started to be consumed, being completely depleted at 24 h. By these results, it is not clear if there was only a sequential consumption of these monosaccharides or if there was also their simultaneous consumption. Fernández-Sandoval et al. [36] studied the same modified *E. coli* strain, MS04, in a batch fermentation with another synthetic medium, using more concentrated sugars, around 50 g/L, and identified three different periods: in the first period, only glucose was consumed; in the second one, the simultaneous consumption of glucose and xylose until glucose depletion took place; and, in the third one, the consumption of the remaining xylose took place.

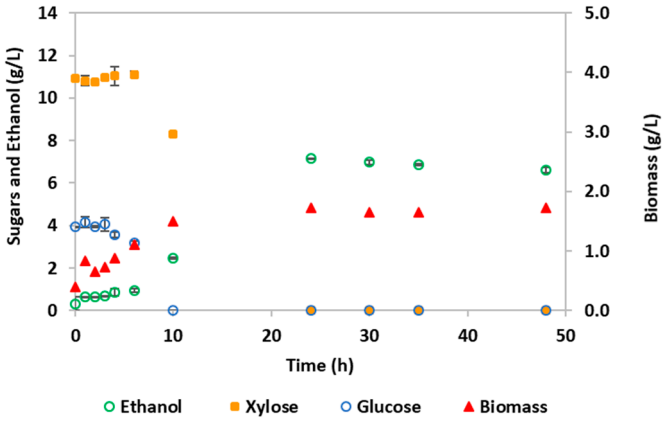

Figure 3. Time course of *E. coli* MS04 fermentation using the hydrolysate detoxified by membrane nanofiltration: glucose, xylose, bioethanol and biomass concentrations (45 mL, 150 rpm, initial pH 7 and 37 °C).

In Figure 3, the maximum bioethanol concentration, i.e., 7.16 g/L, was detected after 24 h of fermentation, when the xylose was depleted. Compared to the same fermentation conditions used for *S. stipitis*, the performance of bioethanol production by *E. coli* MS04 was improved, and an increase from 5.21 to 7.16 g/L (1.4 times) of bioethanol production was achieved. Moreover, an increase in the glucose and xylose consumption rate was observed by *E. coli* MS04, being both sugars completely consumed after 24 h. For *S. stipitis* fermentation, the depletion occurred only after 48 h. Even though the modified *E. coli* strain MS04 shows better results with respect to the production of bioethanol, catabolite repression by glucose is still present, since the simultaneous consumption of xylose could not occur at least from the beginning of bioethanol production, reducing the bioethanol productivity. The modified strain would have been even more effective if this characteristic catabolite repression had have been thoroughly removed.

The control fermentation results with the synthetic LB medium presented in Figure 4 do not show any lag phase, reflecting that the inoculum was grown in the same medium and, thus, presenting a distinct performance. It was found that, contrary to the fermentation using the detoxified hydrolysate, a fast complete glucose depletion was observed until 4 h, instead of 10 h, for the detoxified hydrolysate, starting with the consumption of xylose at this time and ending with its exhaustion detected at 24 h. Additionally, for this control fermentation, the carbon catabolic repression was evident, since glucose inhibited the use of the other carbon source, namely xylose [37]. Regarding bioethanol production, its maximum value of 5.21 g/L occurred after 24 h of fermentation, the period corresponding

to the xylose depletion. Then the bioethanol concentration started to decrease, probably due to its reassimilation for biomass cell maintenance.

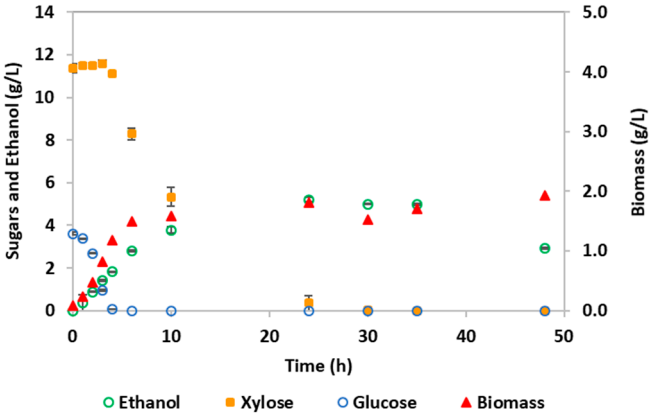

Figure 4. Time course of *E. coli* MS04 fermentation using the LB synthetic medium, glucose, xylose, bioethanol and biomass concentrations (45 mL, 150 rpm, initial pH 7 and 37 °C).

The kinetic and stoichiometric parameters obtained in the fermentations with *E. coli* using the hydrolysate detoxified by membrane nanofiltration and the synthetic LB medium are presented in Table 4. Fermentation carried out with hydrolyzate detoxified with membrane resulted in better outcomes than those obtained with synthetic LB medium, both in the bioethanol yields (0.48 vs. 0.35 g/g) and in the consequent conversion coefficients (94.5 vs. 69.3%). The same applies to the maximum concentrations of bioethanol (7.16 vs. 5.21 g/L) and the consequent maximum productivity values of ethanol (0.30 vs. 0.22 g/L·h). However, none of these differences was found to be statistically significant.

Comparing the parameters with the previous results obtained in the fermentations by *S. stipititis*, it is clear that the fermentation performed by *E. coli* MS04 leads to improved ethanol production. For example, the $Y_{P/S}$ increased from 0.33 to 0.48 g/g with the highest bioethanol level of 7.16 g/L and efficiency conversion of 94.5% for the hydrolysate detoxified with membrane. Additionally, these results indicate that the treated hydrolysate did not affect the bioethanol production, as observed for *S. stipititis*, in which the sugars' consumption and cell growth were affected by the presence of some inhibitory compounds still present in the treated hydrolysate.

Table 4. Kinetic and stoichiometric parameters obtained in the fermentations with *E. coli* MS04 using the synthetic LB medium and the hydrolysate detoxified by membrane.

Parameters [1]	Hydrolysate Detoxified by Membrane Nanofiltration	Synthetic LB Medium	Significance [2]
$Y_{P/S}$ (g/g)	0.48	0.35	NS
Et_{max} (g/L)	7.16 (24 h)	5.21 (24 h)	NS
CE (%)	94.5	69.3	NS
P_{Etmax} (g/L·h)	0.30	0.22	NS

[1] $Y_{P/S}$, ethanol yield; Et_{max}, maximum bioethanol concentration; CE, conversion efficiency; P_{Etmax}, maximum ethanol productivity. [2] NS, not significant.

Since all fermentations with both microorganisms were carried out at similar experimental conditions, it is noteworthy that the genetically modified *E. coli* MS04 has an adapted metabolism for the production of bioethanol. Moreover, the highest production was thoroughly obtained by using the hydrolysate detoxified by membrane nanofiltration, eventually because other carbon staffs could be utilized for bioethanol production.

4. Conclusions

Cardoon hemicellulosic fraction hydrolysate detoxification was compared by both activated carbon adsorption and a nanomembrane filtration system for bioethanol production. It was shown that hemicellulosic fractions of ligninolytic hydrolysates, which are rich in xyloses, C5 sugars, can be used for bioethanol production after a previous efficient detoxification processing. After studding activated carbons adsorption, it was verified that still toxic concentrations of acetic acid (2.71 and 3.25 g/L) were remaining in the hydrolysate not allowing sugars' conversion to bioethanol either by *S. stipitis* or by modified *E. coli* in the studied conditions. Efficient membrane nanofiltration processing allowed the adequate detoxification of lignolytic hemicellulosic hydrolysates (0.04 g/L of acetic acid), since 5.28 g/L of bioethanol was produced by the yeast *S. stipitis.* The modified bacteria *E. coli* MS04 could provide even a higher bioethanol concentration of 7.16 g/L. Process optimization should be further studied in order to implement cardoon biorefinery within the circular economy concept.

Author Contributions: A.P.M.T. analyzed data and wrote the manuscript; M.J.A.G. and T.B. carried out the experiments; M.C.F. supervised the work; G.R.P. carried out the data statistical analysis; A.M.R.B.X. and M.C.F. developed the concept; A.M.R.B.X. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Biofuels Barometer 2020—EurObserv'ER. Available online: https://www.eurobserv-er.org/biofuels-barometer-2020/ (accessed on 5 October 2021).
2. Net Zero by 2050—Analysis-IEA. Available online: https://www.iea.org/reports/net-zero-by-2050 (accessed on 5 October 2021).
3. Branco, R.H.R.; Serafim, L.S.; Xavier, A.M.R.B. Second Generation Bioethanol Production: On the Use of Pulp and Paper Industry Wastes as Feedstock. *Fermentation* **2018**, *5*, 4. [CrossRef]
4. Vergel, O.A.; Cárdenas, D.; García-Contreras, R.; Mata, C. Bioethanol-Diesel Blends Used in Diesel Engines and Vehicles under Transient Operation. In *Bioethanol Technologies*; IntechOpen: London, UK, 2020. [CrossRef]
5. Pereira, S.R.; Ivanuša, Š.; Evtuguin, D.V.; Serafim, L.S.; Xavier, A.M.R.B. Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol. *Bioresour. Technol.* **2012**, *103*, 131–135. [CrossRef] [PubMed]
6. Thamizhakaran Stanley, J.; Thanarasu, A.; Senthil Kumar, P.; Periyasamy, K.; Raghunandhakumar, S.; Periyaraman, P.; Devaraj, K.; Dhanasekaran, A.; Subramanian, S. Potential pre-treatment of lignocellulosic biomass for the enhancement of biomethane production through anaerobic digestion—A review. *Fuel* **2022**, *318*, 123593. [CrossRef]
7. Encinar, J.M.; González, J.F.; Sabio, E.; Ramiro, M.J. Preparation and Properties of Biodiesel from *Cynara cardunculus* L. Oil. *Ind. Eng. Chem. Res.* **1999**, *38*, 2927–2931. [CrossRef]
8. Prussi, M.; O'Connell, A.; Lonza, L. Analysis of current aviation biofuel technical production potential in EU28. *Biomass Bioenergy* **2019**, *130*, 105371. [CrossRef]

9. Pappalardo, H.D.; Toscano, V.; Puglia, G.D.; Genovese, C.; Raccuia, S.A. Cynara cardunculus L. as a Multipurpose Crop for Plant Secondary Metabolites Production in Marginal Stressed Lands. *Front. Plant Sci.* **2020**, *11*, 240. [CrossRef]
10. Espada, J.J.; Villalobos, H.; Rodríguez, R. Environmental assessment of different technologies for bioethanol production from Cynara cardunculus: A Life Cycle Assessment study. *Biomass Bioenergy* **2021**, *144*, 105910. [CrossRef]
11. Fernandes, M.C.; Ferro, M.D.; Paulino, A.F.C.; Mendes, J.A.S.; Gravitis, J.; Evtuguin, D.V.; Xavier, A.M.R.B. Enzymatic saccharification and bioethanol production from Cynara cardunculus pretreated by steam explosion. *Bioresour. Technol.* **2015**, *186*, 309–315. [CrossRef]
12. Amândio, M.S.T.; Rocha, J.M.S.; Serafim, L.S.; Xavier, A.M.R.B. Cellulosic Bioethanol from Industrial Eucalyptus globulus Bark Residues Using Kraft Pulping as a Pretreatment. *Energies* **2021**, *14*, 2185. [CrossRef]
13. Pereira, S.R.; Sànchez i Nogué, V.; Frazão, C.J.R.; Serafim, L.S.; Gorwa-Grauslund, M.F.; Xavier, A.M.R.B. Adaptation of *Scheffersomyces* stipitis to hardwood spent sulfite liquor by evolutionary engineering. *Biotechnol. Biofuels* **2015**, *8*, 50. [CrossRef]
14. Ruchala, J.; Kurylenko, O.O.; Dmytruk, K.V.; Sibirny, A.A. Construction of advanced producers of first- and second-generation ethanol in Saccharomyces cerevisiae and selected species of non-conventional yeasts (Scheffersomyces stipitis, Ogataea polymorpha). *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 109–132. [CrossRef] [PubMed]
15. Brandt, B.A.; Jansen, T.; Volschenk, H.; Görgens, J.F.; Van Zyl, W.H.; Den Haan, R. Stress modulation as a means to improve yeasts for lignocellulose bioconversion. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 4899–4918. [CrossRef] [PubMed]
16. Xavier, A.M.R.B.; Correia, M.F.; Pereira, S.R.; Evtuguin, D.V. Second-generation bioethanol from eucalypt sulphite spent liquor. *Bioresour. Technol.* **2010**, *101*, 2755–2761. [CrossRef] [PubMed]
17. Weissgram, M.; Ters, T.; Weber, H.K.; Herwig, C. Investigating the potential of thermophilic species for ethanol production from industrial spent sulfite liquor. *AIMS Energy* **2015**, *3*, 592–611. [CrossRef]
18. Sarawan, C.; Suinyuy, T.N.; Sewsynker-Sukai, Y.; Gueguim Kana, E.B. Optimized activated charcoal detoxification of acid-pretreated lignocellulosic substrate and assessment for bioethanol production. *Bioresour. Technol.* **2019**, *286*, 121403. [CrossRef] [PubMed]
19. Alves-Ferreira, J.; Carvalheiro, F.; Duarte, L.C.; Ferreira, A.R.P.; Martinez, A.; Pereira, H.; Fernandes, M.C. D-Lactic acid production from Cistus ladanifer residues: Co-fermentation of pentoses and hexoses by Escherichia coli JU15. *Ind. Crops Prod.* **2022**, *177*, 114519. [CrossRef]
20. Arminda, M.; Josúe, C.; Cristina, D.; Fabiana, S.; Yolanda, M. Use of activated carbons for detoxification of a lignocellulosic hydrolysate: Statistical optimisation. *J. Environ. Manag.* **2021**, *296*, 113320. [CrossRef]
21. Peng, P.; Lan, Y.; Liang, L.; Jia, K. Membranes for bioethanol production by pervaporation. *Biotechnol. Biofuels* **2021**, *14*, 10. [CrossRef]
22. Pan, L.; He, M.; Wu, B.; Wang, Y.; Hu, G.; Ma, K. Simultaneous concentration and detoxification of lignocellulosic hydrolysates by novel membrane filtration system for bioethanol production. *J. Clean. Prod.* **2019**, *227*, 1185–1194. [CrossRef]
23. Dos Santos, J.L.C.; Fernandes, M.C.; Lourenço, P.M.L.; Duarte, L.C.; Carvalheiro, F.; Crespo, J.G. Removal of inhibitory compounds from olive stone auto-hydrolysis liquors by nanofiltration. *Desalin. Water Treat.* **2011**, *27*, 90–96. [CrossRef]
24. Fernández-Sandoval, M.T.; Huerta-Beristain, G.; Trujillo-Martinez, B.; Bustos, P.; González, V.; Bolivar, F.; Gosset, G.; Martinez, A. Laboratory metabolic evolution improves acetate tolerance and growth on acetate of ethanologenic Escherichia coli under non-aerated conditions in glucose-mineral medium. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 1291–1300. [CrossRef] [PubMed]
25. Fernandes, M.C.; Ferro, M.D.; Paulino, A.F.C.; Chaves, H.T.; Evtuguin, D.V.; Xavier, A.M.R.B. Comparative study on hydrolysis and bioethanol production from cardoon and rockrose pretreated by dilute acid hydrolysis. *Ind. Crops Prod.* **2018**, *111*, 633–641. [CrossRef]
26. Brás, T.; Guerra, V.; Torrado, I.; Lourenço, P.; Carvalheiro, F.; Duarte, L.C.; Neves, L.A. Detoxification of hemicellulosic hydrolysates from extracted olive pomace by diananofiltration. *Process Biochem.* **2014**, *49*, 173–180. [CrossRef]
27. Vargas-Tah, A.; Moss-Acosta, C.L.; Trujillo-Martinez, B.; Tiessen, A.; Lozoya-Gloria, E.; Orencio-Trejo, M.; Gosset, G.; Martinez, A. Non-severe thermochemical hydrolysis of stover from white corn and sequential enzymatic saccharification and fermentation to ethanol. *Bioresour. Technol.* **2015**, *198*, 611–618. [CrossRef] [PubMed]
28. Snedecor, G.; Cochran, W. *Statistical Methods*, 8th ed.; Iowa State University Press: Ames, IA, USA, 1989; ISBN 0813815614.
29. Zhang, Y.; Li, M.; Wang, Y.; Ji, X.; Zhang, L.; Hou, L. Simultaneous concentration and detoxification of lignocellulosic hydrolyzates by vacuum membrane distillation coupled with adsorption. *Bioresour. Technol.* **2015**, *197*, 276–283. [CrossRef] [PubMed]
30. Ballesteros, I.; Ballesteros, M.; Manzanares, P.; Negro, M.J.; Oliva, J.M.; Sáez, F. Dilute sulfuric acid pretreatment of cardoon for ethanol production. *Biochem. Eng. J.* **2008**, *42*, 84–91. [CrossRef]
31. Palmqvist, E.; Hahn-Hägerdal, B. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. *Bioresour. Technol.* **2000**, *74*, 25–33. [CrossRef]
32. Parajó, J.C.; Domínguez, H.; Domínguez, J.M. Xylitol production from Eucalyptus wood hydrolysates extracted with organic solvents. *Process Biochem.* **1997**, *32*, 599–604. [CrossRef]
33. Hao, X.C.; Yang, X.S.; Wan, P.; Tian, S. Comparative proteomic analysis of a new adaptive Pichia Stipitis strain to furfural, a lignocellulosic inhibitory compound. *Biotechnol. Biofuels* **2013**, *6*, 34. [CrossRef]
34. Günan Yücel, H.; Aksu, Z. Ethanol fermentation characteristics of Pichia stipitis yeast from sugar beet pulp hydrolysate: Use of new detoxification methods. *Fuel* **2015**, *158*, 793–799. [CrossRef]

35. Farias, D.; Maugeri Filho, F. Co-culture strategy for improved 2G bioethanol production using a mixture of sugarcane molasses and bagasse hydrolysate as substrate. *Biochem. Eng. J.* **2019**, *147*, 29–38. [CrossRef]
36. Fernández-Sandoval, M.T.; Galíndez-Mayer, J.; Bolívar, F.; Gosset, G.; Ramírez, O.T.; Martinez, A. Xylose-glucose co-fermentation to ethanol by Escherichia coli strain MS04 using single- and two-stage continuous cultures under micro-aerated conditions. *Microb. Cell Fact.* **2019**, *18*, 145. [CrossRef] [PubMed]
37. Bren, A.; Park, J.O.; Towbin, B.D.; Dekel, E.; Rabinowitz, J.D.; Alon, U. Glucose becomes one of the worst carbon sources for E.coli on poor nitrogen sources due to suboptimal levels of cAMP. *Sci. Rep.* **2016**, *6*, 24834. [CrossRef] [PubMed]