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Improved xylitol production from olive stones hydrolysates by biological detoxification

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Abstract

Olive stones, currently used as a biosource of heat and power, is gaining research interest as a feedstock for a biorefinery based on olive derived wastes. In this work, pure xylitol crystals were obtained and fully characterized as one of the possibilities of taking full advantage of this by-product of olive oil production. The proposed process includes a two-step pretreatment (water and acid extractions), detoxification of the liquid fractions and fermentation of sugars. After the water extraction, a liquid fraction with antioxidant capacity was recovered; the second step was done with dilute sulfuric acid for solubilization of sugars, resulting in a solution containing more than 60 g/l of xylose as the main sugar. This liquor contained also up to 16 g/l acetic acid, which rendered it not fermentable. Also other

compounds, potentially inhibitors in downstream operations, were produced during the pretreatment, requiring a detoxification procedure. In this work, a chemical-free, biological treatment with *Saccharomyces cerevisiae* proved to be the best detoxification method, as glucose, furfural, hydroxymethylfurfural and acetic acid were consumed. The subsequent inoculation of *Candida boidinii* produced xylitol concentrations above 38 g/l after 90h fermentation time and a yield as high as 63%, equivalent to 12 g xylitol/100 g olive stones.

Keywords: Biorefinery, xylitol, olive stones, biological detoxification, green process

1. Introduction

Olive stones (OS) constitute an abundant agro-industrial by-product in the olive oil extraction and pitted table olive industries. OS are a lignocellulosic biomass, therefore mainly composed of hemicellulose (19–32%) cellulose (28–40%) and lignin (25–27%) (Romero-García et al., 2014). Stones represents approximately ten percent by weight of the olive fruit, and they are usually retrieved as crushed small particles from the olive oil production process. Due to the relatively high heating value of OS (20 MJ/kg) (Mata-Sánchez et al., 2013), they are mainly used to produce heat or electricity by direct combustion (Pattara et al., 2010); however, other applications such as use as biosorbent (Blázquez et al., 2014), or raw material for activated carbon production (Spahis et al., 2008) have been also reported. Apart from the current utilisations, OS could be a promising raw material to produce value-added products in biorefinery process (Hernández et al., 2014). Due to its relatively high hemicellulose content that is mainly composed of acetylated xylan, one of the possible products of biorefining of OS is xylitol (Lama-Muñoz et al., 2014). Hemicellulose from OS can be effectively and selectively extracted by dilute acid treatment to obtain a xylose-rich hydrolysate that is a potential substrate for microbial production of xylitol (Lama-Muñoz et al., 2014).

Xylitol is a five-carbon sugar alcohol that has been widely utilized by the food-, pharmaceutical-, cosmetic-, and dental care industries (Ahuja et al., 2020, Ur-Rehman et al.,

2015). In addition, xylitol was identified as one of the 12 most promising bio-based platform molecules in 2004 by the United States Department of Energy (DOE) (Werpy and Petersen, 2014). Xylitol is only obtained from biomass, it has no petrochemical alternative, and it could serve as a renewable raw-material for the sustainable substitution of fossil-based compounds or to produce bio-chemicals with new functionalities for a bio-based economy (Delgado Arcaño et al., 2020, Werpy and Petersen, 2014). The global market of xylitol is expected to reach 266.5 thousand metric tons, with a value above US$ 1 billion by 2022 (Delgado Arcaño et al., 2020). However, industrial-scale production of xylitol is still dominated by the chemical hydrogenation process of xylose derived from lignocellulosic raw materials (Dasgupta et al., 2017). In spite of using renewable raw material, it is considered as a non-environmental friendly and energy-consuming process, and it results in high production costs of xylitol (Baptista et al., 2020, Felipe Hernández-Pérez et al., 2019). It requires extensive xylose-purification steps, harsh reaction conditions, excessive hydrogen supply and a metallic catalyst (Dasgupta et al., 2021), making the whole process energy intensive, dangerous and potentially hazardous to the environment. As an alternative to the chemical method, microbial production of xylitol is becoming more attractive and offers several advantages. Microbial xylitol production (1) does not require extensive xylose-purification process due to its high selectivity, (2) requires mild reaction conditions during the fermentative process step, (3) and facilitates the xylitol recovery and purification steps (Felipe Hernández-Pérez et al., 2019). Fermentative xylitol production can allow lower discharge of chemicals into the environment, low carbon prints, less corrosion of reactors and lower energy consumption (Felipe Hernández-Pérez et al., 2019). Hence, the microbial production of xylitol is a promising strategy to establish an environmentally friendly, sustainable, and green process (Dasgupta et al., 2021). In addition, biotechnological routes for xylitol production can be easily integrated into advanced biorefinery processes, improving the viability and sustainability of biomass-valorising processes and facilitating the development of a bio-based economy (Bedő et al., 2019, Özüdoğru et al., 2019). Microbial conversion of xylose into xylitol can be accomplished by using wild-type bacteria, fungi,

yeasts or recombinant strains (Dasgupta et al., 2017). Yeast has been reported to have the capability to produce the highest yield of xylitol (Mohamad et al., 2015). Among yeasts, Candida species, such as *C. tropicalis* (Singh et al., 2021), *C. guilliermondii* (López-Linares et al., 2018), and *C. boidinii* (López-Linares et al., 2020) are known to be very promising for efficient xylitol production on hemicellulosic hydrolysates. Microbial production of xylitol from hemicellulose hydrolysate is highly influenced by several factors including the microorganism, dissolved oxygen concentration, pH, temperature, initial cell concentration, initial xylose concentration and presence of inhibitor compounds (Bedő et al., 2021). Although dilute acid hydrolysis of olive stone is a well-established, effective method to selectively solubilise the hemicellulose fraction (Lama-Muñoz et al., 2014), the utilisation of the obtained hydrolysate in xylitol fermentation is an actual challenge with restricted information available in the literature. One of the main issues to be solved for effective utilisation of lignocellulosic hydrolysates in xylitol fermentation is the removal of inhibitor compounds obtained during acid hydrolysis (Rafiqul and Sakinah 2013). OS hemicellulose is highly substituted with acetyl groups, hence the acid hydrolysis results in a hydrolysate containing considerable amount of acetic acid that is a strong inhibitor of yeast-mediated xylitol production. The action of acetic acid depends on the concentration of the undissociated form, which is a function of both concentration and pH. Acetic acid is difficult to remove from the lignocellulosic hydrolysates, therefore various separation technologies such as membrane separation (Zhou et al., 2013), chromatography (Nam et al., 2011), emulsion liquid membrane (Lee, 2015), vacuum evaporation (Rodrigues et al., 2003), activated carbon adsorption (Soleimani et al., 2015) and biological treatments (Pereira et al., 2012) have been studied. The use of microorganisms to overcome the effects of inhibitors has also been proposed with the main advantage of no need of chemicals and operation at ambient conditions, constituting a greener approach to detoxification (Parawira and Tekere, 2011). However, the effectiveness of a detoxification process depends both on the composition of hemicellulosic hydrolysate and on the species of microorganism used (Rafiqul and Sakinah, 2013).

The main objective of the present work is the investigation of xylitol production from hemicellulosic hydrolysates of OS using *Candida boidinii* yeast. The effects of acetic acid concentration, initial xylose and cell mass concentrations on xylitol production by *C. boidinii* were investigated on synthetic fermentation medium and the results were evaluated by statistical methods. Different detoxification methods aimed at the removal of acetic acid from the acid hydrolysate of OS were performed and the detoxified liquors were tested in xylitol fermentation under optimised conditions.

2. Materials and methods

2.1. Raw material

Olive stones (OS) were supplied by a local olive-oil mill factory. The stones were separately removed from the olive pomace with an industrial pitting machine, with a 6 mm sieve separator, which is the standard size in this industrial process, soaked in water, washed to free them from any adhering flesh, air-dried and then dried for 24 h at 50°C. Olive stone was characterized in terms of ash (0.5 %), extractives (5.5 %), acetyl groups (4.6 %), moisture content (7.5 %) and lignocellulosic composition: 19.2 % cellulose; 28.6 % hemicelluloses (with xylose accounting for more than 80 % of sugars) and 37.2 % lignin. In addition, OS contain pulp (1.6 %) and skin (1.4 %) residues (Lama-Muñoz et al., 2014).

2.2. Yeast strain and inoculum preparation

Candida boidinii NCAIM Y.01308 was purchased from the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). Yeast strain was maintained on agar slants containing 1% glucose, 1% peptone, 0.3% yeast extract and 2% agar at room temperature. The medium used for inoculum preparation (pH=6) contained 10 g/l yeast extract, 5 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$, 3 g/l $(NH_4)_2HPO_4$ and 30 g/l xylose. The solutions of the xylose and the other components were sterilized separately at 120°C for 20 min in autoclave. Cells were cultivated in 250-ml cotton-plugged Erlenmeyer flasks containing 50 ml inoculum medium at 220 rpm rotation speed in a rotary shaker at 30°C for 72 h (Feher et al.,

2016), subsequently recovered by centrifugation (3500 rpm (1602g) 10 min. Rotina 420, Hettich Zentrifugen, Germany) and the adequate amount of cell mass was directly resuspended in the xylitol fermentation medium.

2.3. Experimental procedure

Figure 1 shows the outline of the experimental procedure performed in this work. Briefly, olive stones were submitted to aqueous extraction and the resulting solids were further treated with dilute sulphuric acid. The liquid fraction issued from this step, together with the washing water of the pretreated solids were used as fermentation media to produce xylitol following different detoxification procedures. Optimal fermentation conditions were previously determined through an experimental design. Details for each step are given next.

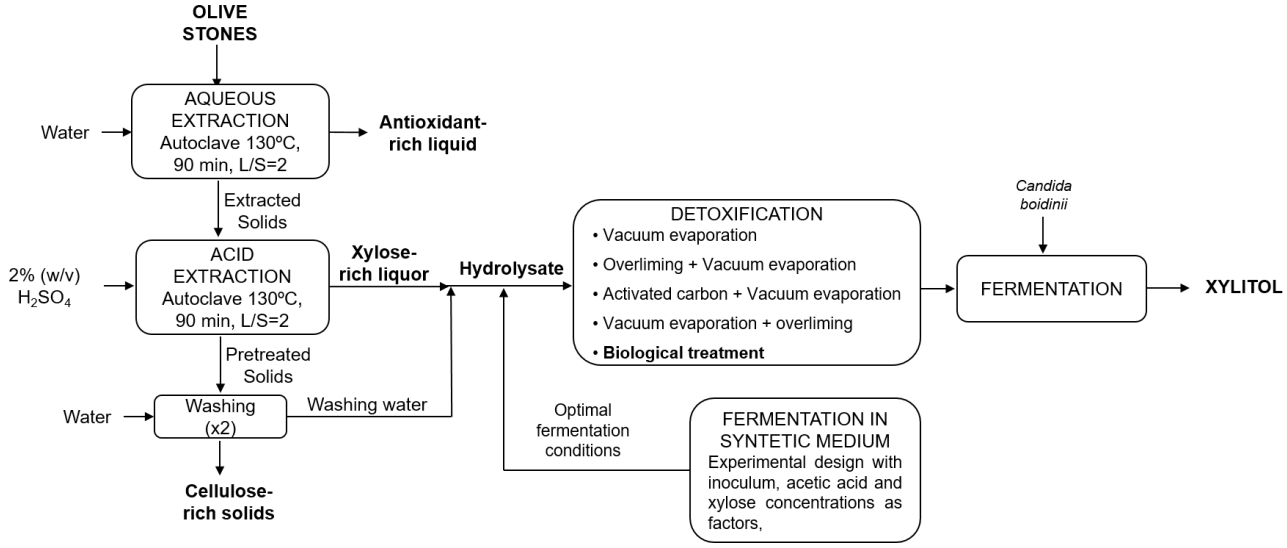


Figure 1. Scheme of the experimental procedure performed

2.3.1. Pretreatment in autoclave

OS were submitted to a two-step process under optimized conditions to recover antioxidants in the first step and to release fermentable sugars in the second step, allowing to make a more integral use of olive stone from the point of view of a biorefinery plant (Lama-Muñoz et al., 2014).

Crushed OS (150 g on dry basis) were loaded into a one-liter glass bottle. The first step consisted of an aqueous extraction at 130 °C for 90 min and a solid-liquid ratio 1:2 (w/w). In

the second step, wet olive stones were subjected to a treatment with 2 %(w/v) sulfuric acid (130 °C for 90 min and solid-liquid ratio 1:2 (w/w)) to obtain the maximum amount of fermentable sugars. As OS is a very porous material, the solid fraction was washed out with 200 mL of water (in two steps at 30 °C for 10 min each time, 220 rpm orbital shaker) for removing impregnated liquid, and then filtered and oven-dried for determination of the total gravimetric recovery. Finally, both the pretreatment liquid fraction and water used for washing were mixed together, being referred to as hydrolysate, and used for further processing.

2.3.2. Experimental design for fermentation of synthetic liquor

The effect in yields and volumetric productivity of three main factors on the fermentation with *Candida boidinii* for xylitol production was studied, e.g., initial concentration of acetic acid (in the range 0-12 g/l), xylose (30-90 g/l) and inoculum concentration (1-5 g/l). A Box-Behnken experimental design, with 17 runs including twelve conditions and five replicates at the midpoint of the factors was used, as shown in Table 1. 50 mL of synthetic liquor together with the cell mass was introduced into a 100-mL Erlenmeyer flasks closed with cotton plugs (microaerobic conditions, (Feher et al., 2016))**.** The synthetic liquor contained inoculum medium salts (10 g/l yeast extract, 5 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$, 3 g/l $(NH_4)_2HPO_4$) and was adjusted to pH=6 with KOH. The fermentation was carried out at 30 °C and 150 rpm in an orbital shaker. Samples were taken every 24 hours, analyzed by HPLC to determine the content of xylose, acetic acid, ethanol and xylitol and the optical density was measured to determine cell concentration.

Experimental data were analyzed via response surface methodology by the statistical software Design-Expert 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA). This analysis allowed for selecting the optimal fermentation conditions to obtain the highest xylitol yield and to be able to apply these conditions to the fermentation of the hydrolysate.

Table 1. Experimental design to study the effects of the initial concentration of xylose, acetic acid and cells during fermentation with *Candida boidinii* for xylitol production

Run	[Cells] (g/l)	[Xylose] (g/l)	[Acetic acid] (g/l)
1	3	90	12
2	3	60	6
3	3	60	6
4	3	30	0
5	1	60	0
6	5	30	6
7	5	60	0
8	3	30	12
9	1	90	6
10	1	30	6
11	3	60	6
12	3	90	0
13	5	90	6
14	3	60	6
15	5	60	12
16	3	60	6
17	1	60	12

2.3.3. Detoxification

Different detoxification methods, individually and combined, were use with the OS liquor obtained in the autoclave, summarized next:

1) Vacuum evaporation: liquor was evaporated for about 1 hour at a pressure of 74-80 mbar and 55°C, to prevent the formation of inhibitor compounds, obtaining liquor concentrate about 7 times, at an evaporation rate of about 2.5 g/min (Rotavapor R-210, BÜCHI, Switzerland).

2) Overliming+Vacuum evaporation: an overliming procedure based on the reports by (Martínez et al., 2000) was applied to the liquor by adding $Ca(OH)_2$ until pH 10. Then the liquor was agitated in an orbital shaker for 45 min at 50ºC and 200 rpm, and finally it was centrifuged at 5000 rpm (3270g) for 5 min (x2) for solid separation (Rotina 420, Hettich Zentrifugen, Germany). Finally, vacuum evaporation was carried out with the above conditions.

3) Activated carbon+Vacuum evaporation: an activated carbon procedure based on the reports by (Mussatto S.I. and Roberto I.C., 2004). The liquor was mixed with activated carbon (Sigma-Aldrich, 100 mesh) with a solid/liquid ratio of 5 %(w/v). Then the liquor was agitated in an orbital shaker for 45 min at 50ºC and 200 rpm and finally it was centrifuged at 5000 rpm (3270g) for 5 min (x2) (Rotina 420, Hettich Zentrifugen, Germany) and filtered through 0.45 µm membranes for solid separation. Lastly, vacuum evaporation was carried out with the conditions described above.

4) Vacuum evaporation+overliming: first, liquor was evaporated. Then, concentrate liquor was resuspended in distilled water in the same volume evaporated. Finally, the liquor was subjected to the overliming procedure.

5) Biological treatment: *Saccharomyces cerevisiae* was utilized. Yeast was in lyophilized form (Ethanol Red, Lesaffre, France). The medium used for inoculum preparation (pH=5.5) contained 5 g/l yeast extract, 2 g/l NH_4Cl, 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \times 7H_2O$ and 30 g/l glucose. The solutions of the glucose and the other components were sterilized separately at 120°C for 20 min in autoclave. Cells were cultivated in 250-mL cotton-plugged Erlenmeyer flasks containing 50 ml inoculum medium at 220 rpm rotation speed in a rotary shaker at 30°C for 24 h, subsequently recovered by centrifugation (3500 rpm (1602g), 10 min. Rotina 420, Hettich Zentrifugen, Germany) and the adequate amount of cell mass was directly resuspended in the liquor. The liquor was detoxified with 5 g/l of cells and cultured under the same growth conditions (initial pH=5.5, 30 °C, 220 rpm). After the time required for eliminating acetic acid has elapsed, the liquor is centrifuged to remove the cells.

2.3.4. Fermentation experiments

Each fermentation liquor was adjusted to pH=6 under sterile conditions with KOH or H_2SO_4 according to its initial pH. Fermentations with *Candida boidinii* were carried out at 30 ºC in 100-mL Erlenmeyer flasks closed with cotton plugs under microaerobic conditions (0.5 filling ratio at 150 rpm) (Feher et al., 2016). The adequate amount of cell mass was directly resuspended in the liquor. Samples were taken daily, analyzed by HPLC to determine the

content of sugars, inhibitors compounds, ethanol and xylitol and the optical density was measured to determine cell concentration.

2.4. Xylitol crystallization

After fermentation, cells were removed from the broth by centrifugation (6000 x g). Cell-free supernatant was treated with 5% w/w activated carbon (Cabot DX Ultra 8003.4, Norit) for 1 hour at room temperature (25°C) with continuous agitation. Then, the activated carbon and the clarified supernatant were separated by filtration through pleated filter paper. The clarified supernatant was treated with 10 % w/w ion-exchange resins (mixed anion- and cation-exchange resins, Ion-Resin-1, Pure Resin Co., Ltd.) for 30 minutes at 30°C with continuous shaking (220 rpm). Ion exchange treatment reduced the conductivity of the clarified supernatant from 11910 µS/cm to 39 µS/cm. The ion exchange resin was separated by filtration using paper filter. Then, the de-ionized liquid fraction was concentrated until a xylitol concentration of 720 g/L by vacuum evaporation at 65°C. The concentrated liquid was cooled down to 4°C in refrigerator for one day, then it was mixed with ethanol (96% v/v) in a volume ratio of 1:1 and seed xylitol crystals were added. It was kept at 4°C in refrigerator for 3 days. Xylitol crystals were then separated by filtration using filter paper, washed with pure ethanol and then dried at room temperature (25°C).

2.5. Analytical methods

2.5.1 Characterization of raw material, pretreated solids, hydrolysates and cells

The composition of raw material and other solids and the composition of hydrolysates obtained from pretreatment and other liquors (monomeric sugars, oligomeric sugars, the total phenolic content, etc.) was determined according to the procedure described by Fernandes-Klajn et al (2018). Analytical methods for biomass of National Renewable Energy Laboratory of the United States were used to determine the chemical composition of raw material and solid samples generated. The content of glucose, xylose, galactose, arabinose, furfural,

HMF, formic acid, acetic acid, levulinic acid, ethanol and xylitol was analysed by HPLC. The total phenolic content of liquid was determined using Folin-Ciocalteu's reagent method. After one hour, the absorbance was measured at 765 nm and a calibration curve for gallic acid was constructed. The results of the total phenolic content in the hydrolysates were expressed as g of gallic acid equivalents (GAE).

Cell concentration was calculated from the optical density of the sample using an appropriate calibration curve. Optical density was determined by spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at a wavelength of 600 nm (Feher et al., 2016).

2.5.2 Analysis of xylitol crystals

Xylitol crystals were analysed by thermogravimetry (TG), X-ray diffraction (XRD) and attenuated total reflection fourier transform infrared (ATR FT-IR) spectroscopy. Measurements were performed on the crystals produced by fermentation (fermented xylitol), xylitol purchased from a local market (commercial xylitol), and analytical grade xylitol (Sigma X3375-25G) purchased from Sigma (standard xylitol). Images from the xylitol crystals were produced by stereo microscope (Nikon SMZ800) at a magnification of 63x.

The TG characterization was performed using a Perkin Elmer TGS-2 thermobalance used with a modified furnace and a temperature controller (Eurotherm, UK). Approximately 2.5 mg of xylitol samples were measured in a platinum sample pan under argon atmosphere at a flow rate of 140 mL/min. The furnace was heated at a rate of 20 °C/min from room temperature to 900°C (Babinszki et al., 2021).

Powder XRD patterns were recorded with a PANalytical X'pert Pro MPD (PANalytical Bv., The Netherlands) multipurpose X-ray diffractometer using Cu K_α radiation, Ni filter, X'celerator detector, and „top-loaded" sample holders in the range of 2θ = 4–44 °. Applied tube voltage and current was 40 kV and 30 mA, respectively (Szeleczky et al., 2016).

ATR FT-IR spectroscopic measurements were performed at room temperature (25◦C). FT-IR absorbance spectra of the xylitol samples were scanned from 4000 to 650 cm^{-1} in attenuated total reflectance (ATR) mode by using a Spectrum 400 spectrometer equipped with a Universal ATR (UATR) accessory (PerkinElmer Inc., Waltham, MA, USA) which contains a single reflection diamond/ZnSe composite crystal. One hundred N force was applied using a pressure arm equipped with a cylindrical “flat shoe” part to delivering sufficient physical contact between the xylitol samples and the ATR crystal during the spectral data collecting, which was performed with Spectrum 6.3.2 software (PerkinElmer Inc., Waltham, MA, USA) (Szabó et al., 2020).

3. Results and discussion

3.1. Olive stone pretreatment in autoclave

The composition of solids and liquids produced after the OS two-step pretreatment in autoclave are summarized in Table 2 and Table 3. The first step provided the double benefit of separating phenolic compounds, whilst also saving biomass of possible inhibitors to enhance further fermentation steps.

Concerning the solid fractions, raw OS contained approximately 53 g of sugars per 100 g OS, with xylose as the main one, and 37% lignin. After the antioxidant extraction step, more than 97.8% of initial solid was recovered and only a small increase of xylose content was detected. In the sugars extraction step, after pretreatment (extracted OS) with H_2SO_4 (2 %w/v), OS became more porous whereby a wash step is necessary to recover a larger amount of sugar.

Table 2. Summary of the composition (g/100 g) of initial OS and solids obtained after the two step pretratment process

	Initial OS	Solid after aqueous extraction	Solid after acid extraction
Glucose	21.16±0.51	21.23±0.42	33.24±1.00
Xylose	27.13±0.31	29.32±0.88	3.75±0.08
Galactose	2.80±0.04	3.67±0.07	nd

Arabinose	2.63±0.04	2.02±0.06	nd
Lignin	37.18±0.46	37.28±0.75	54.94±1.10
Acetic acid	6.56±0.06	5.98±0.06	0.64±0.01

nd: not detected

As far as the liquid fraction is concerned, less than 1.5% of the initial sugars (0.79 g/100g OS) was recovered, from which more than 70% are in oligomeric form (0.58 g/100g OS). The oligomers were mainly composed by hemicellulose sugars (mainly arabinose). It is worth noting that the total phenolic content was 372 mg GAE/100g OS, representing this amount over 96% of the estimated by (Lama-Muñoz et al., 2014) (387 mg GAE/100g OS), who concluded that OS extracts in these conditions had a similar antioxidant capacity to BHT (Butylhydroxytoluene, E321), a synthetic antioxidant widely used in the food industry. Table 3 shows that the liquor obtained after the acid pretreatment had a concentration of sugars (sum of glucose, xylose, galactose and arabinose) of 114.8 g/l, with xylose accounting for more than 83% (95.7 g/l). In the OS washing step, a liquor with a total sugar concentration above 33 g/l and a xylose content of more than 28 g/l was obtained. The liquor produced by mixing of acid pretreatment liquor and washing liquor (referred to as “hydrolysate”) had a sugar concentration of 74.4 g/l, with a xylose concentration of 64.9 g/l. The composition of these liquors is very interesting for the production of xylitol because xylose is the main component, above 80%, which facilitates purification and crystallization of xylitol.

The total recovery of sugars in the acid pretreatment and washing were 23.2 g/100g OS, almost 90% of the estimated by (Lama-Muñoz et al., 2014) (26.6 g/100g OS), which is equivalent to 44.2% total initial sugar content. In the washing step, more than 21% of total sugars (acid pretreatment+washing) was recovered (4.9 g/100g OS). The sugars recovery yield in the washing step is about 65% of the sugars present in the liquid retained in the pretreated OS. In the hydrolysate, 68.5% of the hemicellulosic sugars (sum of xylose, galactose and arabinose) was recovered. Xylose was the most recovered sugar, with almost 71%, while less than 7% of initial glucose was recovered. Recovery of the solid after the process is 68.8% and this solid is rich in cellulose (30.2%) and lignin (54.9%), which opens

many possibilities as an interesting feedstock for the recovery of lignin and glucose content (Rodríguez-Gutiérrez et al., 2014).

Table 3. Composition (g/L) of the liquid fractions obtained from the two-step pretreatment of OS

	Liquor from aqueous extraction step		Liquor from acid extraction step		
	Liquid (g/l)	Oligomers in liquid (g/l)	Xylose-rich liquor (g/l)	Washing water (g/l)	Hydrolysate (g/l)
Glucose	0.53±0.01	0.41±0.01	6.52±0.20	1.63±0.02	3.16±0.6
Xylose	0.02±0.00	0.66±0.01	95.75±0.96	28.30±0.57	64.91±1.95
Galactose	0.10±0.00	0.54±0.02	8.00±0.08	2.10±0.02	4.14±0.12
Arabinose	0.72±0.02	2.08±0.06	4.58±0.14	1.14±0.01	2.51±0.08
Acetic acid	1.04±0.03		24.34±0.24	8.78±0.02	16.77±0.34
Formic acid	0.45±0.01		2.47±0.07	0.84±0.02	1.64±0.02
Levulinic acid	nd		0.02±0.00	0.03±0.00	0.02±0.00
Furfural	nd		3.71±0.04	1.68±0.03	2.73±0.08
HMF	nd		0.06±0.00	0.02±0.00	0.04±0.00
Total phenols*	1.86±0.04				

nd: not detected

*(g GAE/L)

Regarding the content of inhibitor compounds of the fermentation is noteworthy acetic acid accounts with almost 80% of total inhibitors measured with concentrations between 8.9 and 24.3 g/l. According to several authors, concentrations of acetic acid higher than 1.1, 1.2 and 3.0 g/l may affect negatively the production of xylitol by *C. guilliermondii*, *C. tropicalis* and *C. parapsilosis,* respectively (Pereira et al., 2011, Wang et al., 2013).

3.2. Fermentation of synthetic liquor

Preliminary tests performed on both the liquor issued from the acid extraction step and the mixture of it with the washing water (hydrolysate) revealed a complete inhibition of growth of *C. boidinii*, which was attributed to the high concentration of acetic acid in these liquors (Table 2). Because of it, an experimental design was applied to determine the best conditions for the fermentation of the hydrolysates, considering acetic acid, xylose and inoculum concentrations as the main factors.

Figure 2 shows the influence of these factors on the growth of *C. boidinii*. In all cases, an increase in cell concentration (higher in the case without acetic acid) was observed, despite

the micro-aerobic conditions applied during the fermentations. The increase in the cell concentration was greater at smaller initial concentrations of cells. Similar behaviour was observed during xylitol fermentation on semi-defined medium with *C. boidinii* by Feher et al. (2016). Where the initial cell concentrations were 1 and 3 g/l, a very negative effect of the presence of acetic acid was observed, resulting in 60% less formation of cells at the end of fermentation. For the initial cell concentration of 5 g/l, the presence of acetic acid did not exert a significant effect during 100h, thereafter it showed a negative effect with a reduction of around 25% in the formation of cells. The initial concentration of xylose did not result in a significant effect on the formation of cells. In terms of xylitol formation, high increase in the cell concentration during the fermentation is disadvantageous due to the consumption of xylose that supposed to be converted into xylitol.

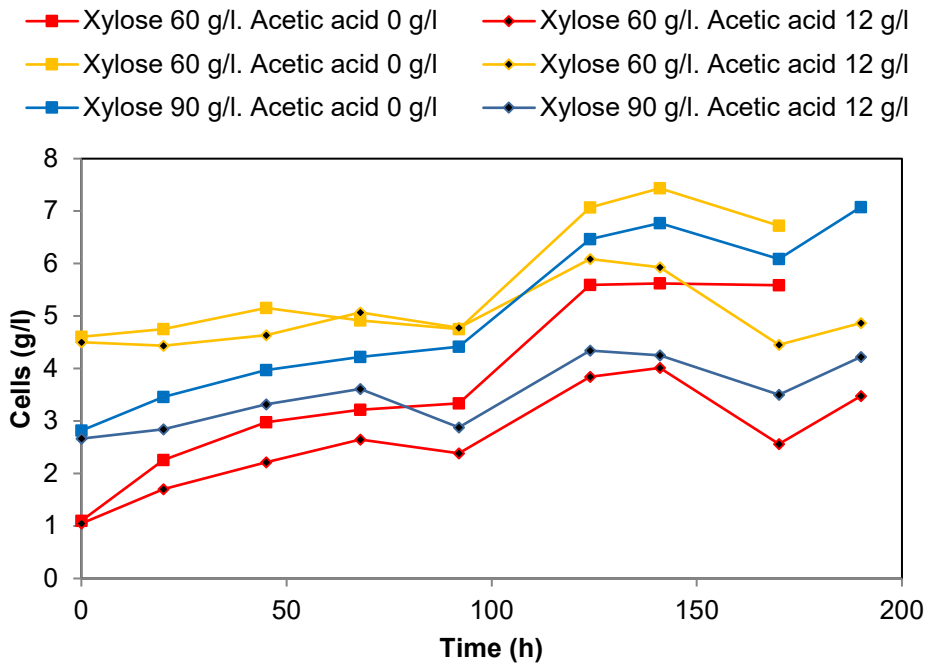


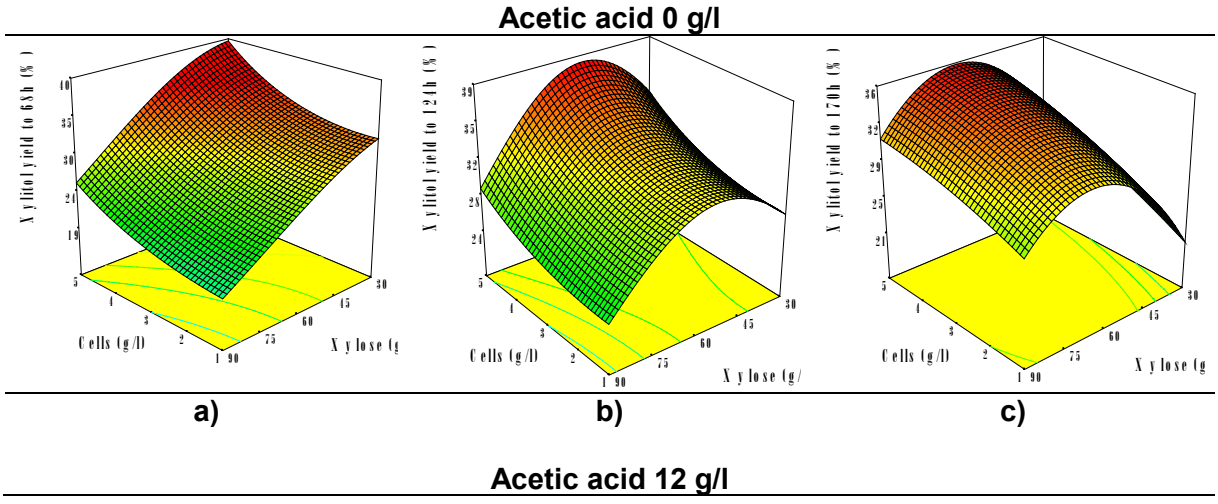
Figure 2. Evolution of the *Candida boidinii* cell concentration under different conditions of initial concentrations of xylose, acetic acid and cells studied in synthetic liquor fermentation

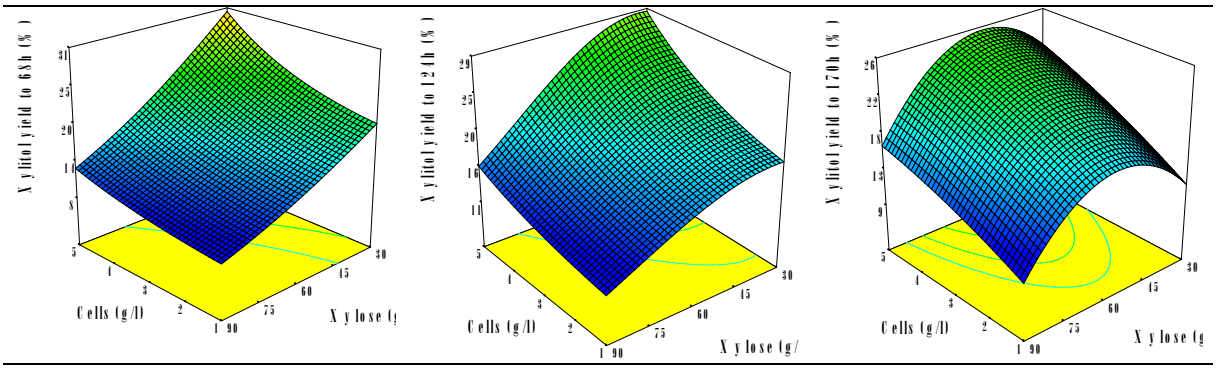
In Figure 3 the influence of the three factors studied (initial concentration of xylose, cell and acetic acid) on the xylitol yield with *C. boidinii* at different fermentation times is shown. The most influential factor was the initial concentration of acetic acid, having a very negative effect which reduces the final xylitol yield by about 25% when increasing the acetic acid

concentration from 0 to 12 g/l. The above behaviour was observed in the entire range of initial concentration of xylose and cells and for all fermentation times studied. As it was mentioned earlier, many studies on xylitol production by yeasts belonging to the genus of *Candida* indicated that acetic acid can have a strong inhibitory effect on xylitol production (Pereira et al., 2011, Wang et al., 2013).

The initial concentration of xylose was the next most influential factor, changing its effect with fermentation time. Increasing the concentration of xylose had a negative effect on the xylitol yield at short fermentation times (Figure 3.a,d), and for medium and long times presented a quadratic behaviour with a peak (Figure 3.b,c,e,f). The negative effect of high initial xylose concentration on the xylitol production of *C. boidinii* was also observed by Vandeska et al (1995), which might be due to osmotic stress on the cells.

Finally the initial concentration of cells was presented as the least influential factor, taking in all cases the best xylitol yields with the highest concentration of cells. Application of high initial cell concentration (around 5 g/l) in xylitol fermentations was found to be favourable in previous studies with *C. boidinii* (Vandeska et al, 1995).





d) **e)** **f)**

Figure 3. Response surfaces of xylitol yield based on the initial concentrations of xylose and cells at different times of synthetic liquor fermentation with *Candida boidinii*. Without acetic acid: a) 68h, b) 124h, c) 170h; 12 g/l acetic acid: d) 68h, e) 124h, f) 170h (all cases R^2>0.99)

Analogously to the xylitol yield behaviour, regarding the volumetric productivity, acetic acid was the most influential factor and its effect is negative. In this case the influence was even higher since the reduction in the xylitol volumetric productivity was about 50% when increasing the concentration of acetic acid from 0 to 12 g/l (Figure 4). The initial concentration of xylose is the second most influential factor showing a positive effect with the increase in the initial concentration, the same trend as with the initial concentration of cells, the latter being the least influential factor. The volumetric productivity and yield of xylitol have been optimized together at different fermentation times; in all cases the maximum initial cell concentration (5 g/l) and the minimum initial acetic acid concentration (0 g/l) is considered (Table 4). The optimal xylose initial concentration changes with the fermentation time being between 55 and 76 g/l for times between 68 and 170h respectively. This increase in the initial optimal xylose concentration with increasing fermentation time can be attributed to the fact that at longer times the xylose is almost completely consumed with the lower initial xylose concentrations, and therefore the volumetric productivity of the xylitol is very low (Figure 4.c,f). The largest volumetric productivity of xylitol was obtained in shorter fermentation time (68h), as expected, while the highest xylitol yield was obtained at intermediate time of fermentation (124h) with an optimum of xylose initial concentration at 60 g/l.

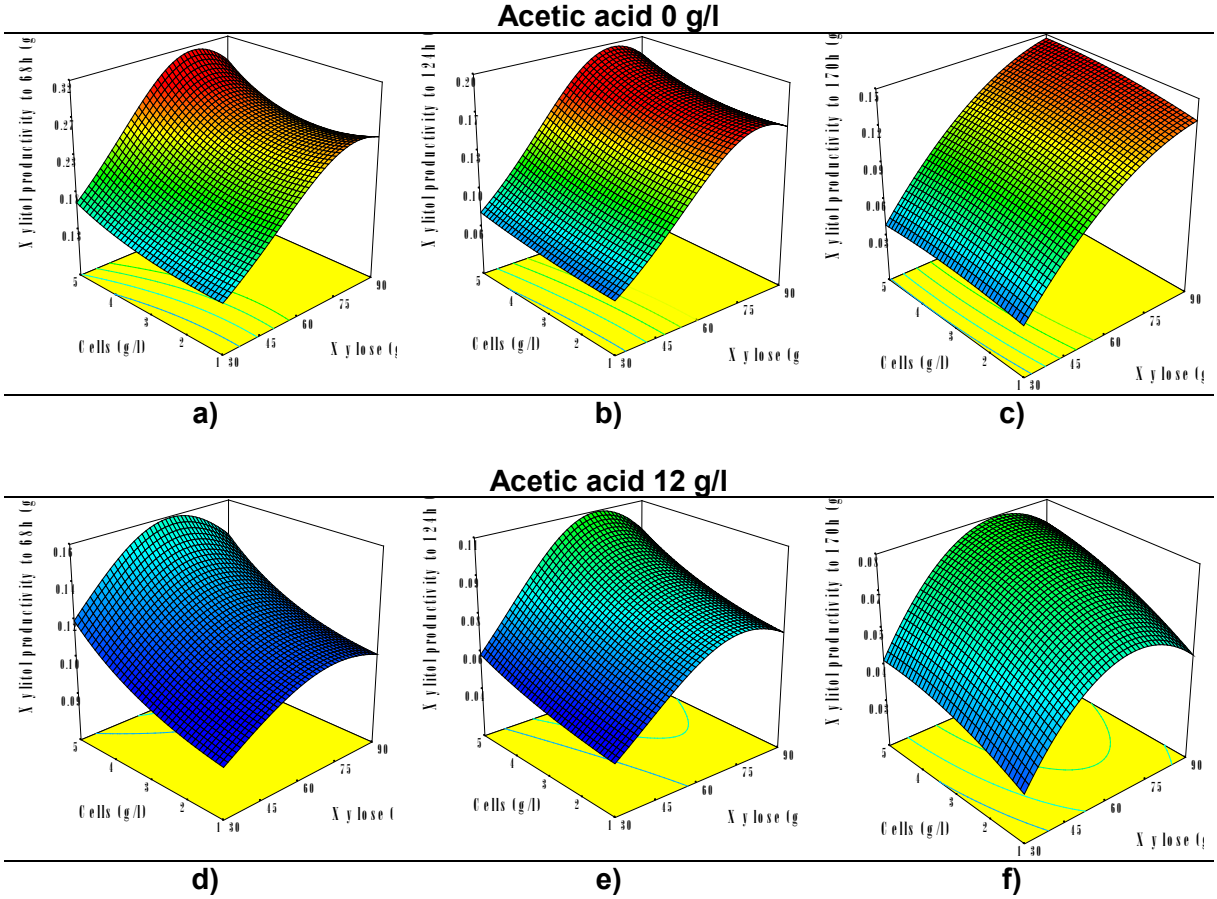


Figure 4. Response surfaces of xylitol volumetric productivity according to the initial concentrations of xylose and cells at different times of synthetic liquor fermentation with *Candida boidinii*. Without acetic acid: a) 68h, b) 124h, c) 170h; 12 g / l acetic acid: d) 68h, e) 124h, f) 170h (all cases R^2>0.99)

Table 4. Co-optimization of volumetric productivity and yield of xylitol with different fermentation times with *C. boidinii* in synthetic liquors

Fermentation time (h)	Cells (g/l)	Xylose (g/l)	Acetic acid (g/l)	Xylitol yield (%)	Xylitol productivity (g/l/h)
68	5	55.91	0	35.70	0.27
124	5	60.29	0	37.98	0.17
170	5	76.53	0	34.62	0.14

3.3. Hydrolysates detoxification

From the results presented above and those described by other authors for different xylitol producer microorganisms (*C. guilliermondii*, *C. tropicalis, C. parapsilosis,* etc.), it can be deduced that the detoxification of the hydrolysate obtained is necessary before fermentation to reduce the concentration of acetic acid and other inhibitor compounds (furfural, HMF, etc.) (Pereira et al., 2011, Wang et al., 2013) In our case, acetic acid represents about 80% of the

inhibitor compounds measured. The hydrolysate, mixture of the pretreatment liquor and wash liquor (Table 2), was selected because its xylose concentration (~65 g/l) is in the optimum range for xylose initial concentration found for xylitol production (Table 4).

In Figure 5, the recovery of sugar, inhibitor compounds, and total phenols after detoxification with different physic-chemical methods used is summarized. The best recovery of sugars was obtained with evaporation method (>96%); in all cases the recovery of sugars was greater than 86%. The greatest reduction of inhibitory compounds occurred in the combined method of active carbon and vacuum evaporation treatments, where more than 93% were removed. Furfural was completely eliminated in all cases as it is a volatile compound in the evaporation conditions used, while the HMF (low concentration) was removed only in the cases of active carbon treatment or overliming (non-volatile in these conditions). The levulinic acid was the one with a smaller reduction but it must also be emphasized that it is the one with a lower concentration (0.02 g/l). Formic acid has a reduction in all cases of about 60%. Acetic acid removal was greater than 90%. Being a volatile compound, vacuum evaporation works well, but it is worth noting that the effect when combined with overliming depends on the order, i.e., overliming used after vacuum evaporation resulted in acetic acid concentration reduction of almost 92% while if evaporation was placed first, the removal of acetic acid was only 10%. This different result can be attributed to pH, since in the first case, evaporation is very acidic (<1) while in the second case it is basic (>9) (Rodrigues et al., 2001). Finally, with respect to the total phenols, significant reduction occurred when the combination of activated carbon and vacuum evaporation was used, reaching almost 80%.

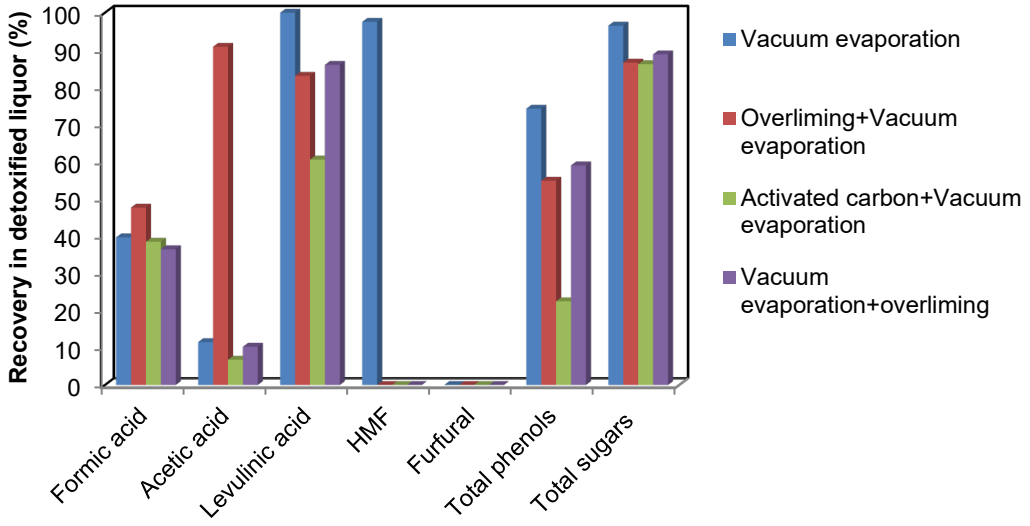


Figure 5. Recovery of sugars, inhibitor compounds (formic acid, acetic acid, levulinic acid, HMF, furfural) and total phenols in detoxified liquor by vacuum evaporation, vacuum evaporation+ overliming, vacuum evaporation+ activated carbon and overliming+ vacuum evaporation

The evolution of the concentration of the inhibitor compounds and sugars during the biological detoxification with *S. cerevisiae* is depicted in Figure 6. *S. cerevisiae* initial concentrations lower than 5 g/l were tested but no reduction occurred in the inhibitor compounds (results not shown). Furfural, HMF and levulinic acid were completely consumed in the first 20h; instead, the formic acid concentration is almost constant, experiencing a slight decrease once all the acetic acid is consumed around 115h. At this time, the maximum concentration of cells around 6.5 g/l was reached and a reduction about 50% of total phenols also occurred. The complete consumption of glucose and other reducing sugars took place, which is positive since it can reduce the cost of purification of xylitol (Nigam and Singh, 1995); and also consumed xylose is converted to xylitol with near 80% yield.

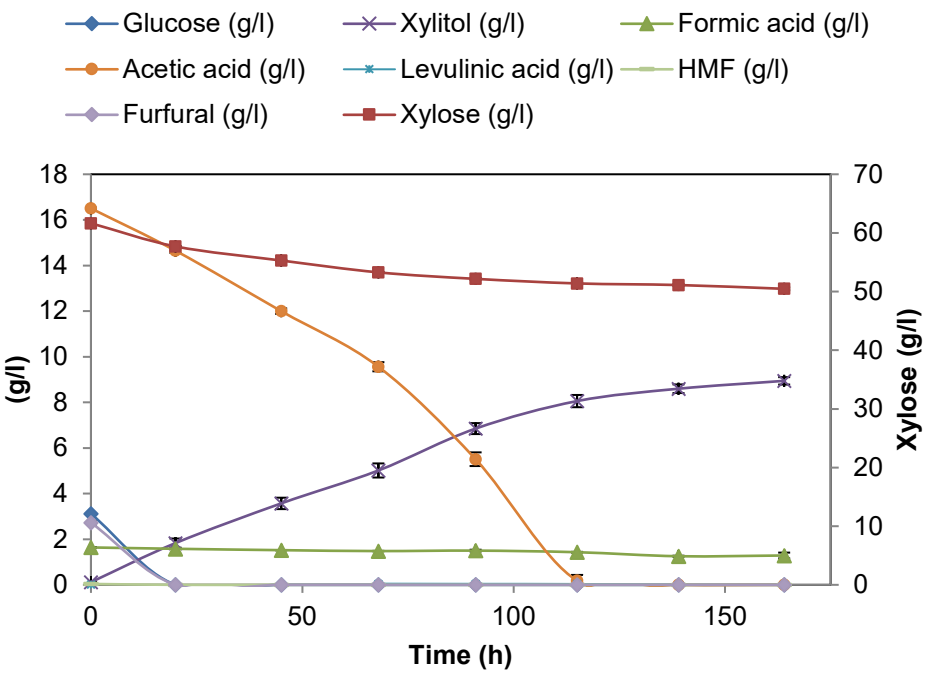


Figure 6. Time course of the concentrations of sugars (glucose and xylose), xylitol and inhibitor compounds (formic acid, acetic acid, levulinic acid, HMF, furfural) in the biological detoxification with *S. cerevisiae*

3.4. Fermentation of hydrolysates

Figure 7 shows the time evolution of the concentrations of xylose (major sugar), acetic acid (majority inhibitor compound), xylitol and ethanol during fermentation with *C. boidinii* of OS detoxified hydrolyzate by the five methods studied (vacuum evaporation, vacuum evaporation+ overliming, active carbon+ vacuum evaporation, vacuum evaporation+ overliming and biological treatment with *S. cerevisiae*) and without detoxification to produce xylitol. No xylose consumption and no xylitol or ethanol production was observed beyond 250 hours of experiment, hence it can be concluded that *C. boidinii* is unable to ferment the OS hydrolysate without detoxification, as occurs with other hydrolysates from olive biomass such as extracted olive pomace (Brás et al., 2014). The hydrolysate without detoxification contained more than 16 g acetic acid/l, although this is not of the reason of the loss of fermentative capacity of the microorganism since, in the case of liquor detoxified with overliming + vacuum evaporation, the acetic acid concentration was around 15 g/l and xylitol was produced (Figure 7.b,c). It is assumed that other inhibitory compounds (e.g. phenolic

substances) can act synergistically with acetic acid and can cause the total inhibition of fermentative capacity when OS hydrolysate was not detoxified (Parajo et al, 1998).

The only case in which xylose was completely consumed (after about 150 h fermentation) was when the hydrolysate was detoxified biologically, which is very positive for the subsequent step of crystallization (Faneer et al., 2018). For comparison, when overliming or activated carbon were used as detoxification procedures, about 7 g xylose/l remained after 250h of fermentation, while it was more than 17 g xylose/l when using only vacuum evaporation (Figure 7.a).

In terms of the formation of xylitol, which is the aim of the fermentation, the highest concentration was obtained with the biological treatment, reaching more than 38 g/l at around 100h. This is the highest concentration obtained so far using olive stone as raw material, comparing favourably with 8.2 g/l previously reported (Saleh et al., 2014) and the highest obtained using other olive-derived wastes, such as extracted olive pomace (5.1 g/l (Brás et al., 2014)) or olive tree pruning biomass (8 g/l (Mateo et al., 2014)). With the other four methods of detoxification used, the final concentration of xylitol was around 23 g/l (Figure 7.c).

During the fermentation with *C. boidinii,* a small amount of ethanol was also produced regardless of the method of detoxification, (Figure 7.d). In all cases, *C. boidinii* produced a small quantity of ethanol during part of the fermentation which was also consumed later, as other xylitol producer microorganisms do (Ko et al., 2008).

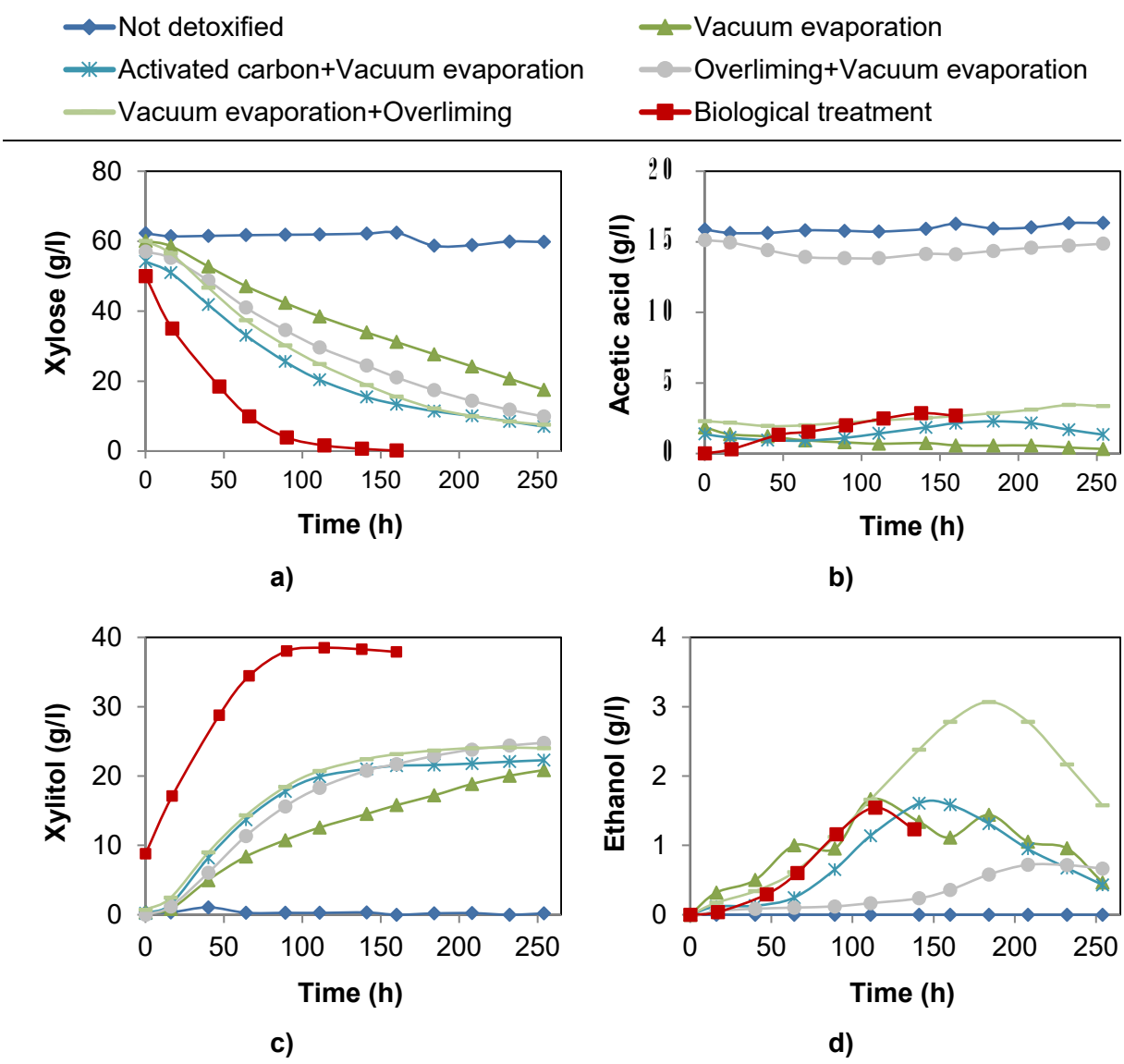


Figure 7. Fermentation with *C. boidinii* of olive stone hydrolyzate detoxified by different methods for production of xylitol: vacuum evaporation, overliming+ vacuum evaporation, activated carbon+ vacuum evaporation, vacuum evaporation+ overliming and biological treatment with *S. cerevisiae*. Evolution of the concentrations of: a) xylose; b) acetic acid; c) xylitol; d) ethanol

In all detoxified hydrolysate fermentations the maximum xylitol yield was obtained at around 50 h of fermentation; then, the yield decreased with fermentation time (Figure 8.a).

The xylitol yield in the detoxified hydrolysate with biological treatment resulted greater than 63% at 90h, which corresponds to the xylitol concentration of 38 g/l. In other detoxified hydrolysates, xylitol yields varied around 50% (maximum xylitol about 23 g/l, at 254 h), similar to the 53% obtained with activated carbon detoxified corn fibre hydrolysate also

fermented with *C. boidinii* (Feher et al., 2016) and 54% obtained with activated carbon+ vacuum evaporation detoxified sugarcane bagasse hydrolysate fermented with *C. guilliermondii* FTI 20037 (Rodrigues et al., 2003). The xylitol yield achieved in this work is the highest achieved so far with olive stone (44% (Saleh M. et al., 2014)) and superior to that obtained with other olive biomass like olive tree pruning biomass (49%, García et al., 2011) and extracted olive paste (26%, Brás et al., 2014).

The behaviour of the xylitol volumetric productivity is very similar to that of the yield, with the maximum around 50h except for the biological treatment in which it is before 24h (Figure 8.b). Xylitol productivity was more affected by the detoxification method than the yield; its maximum value is in the range of 0.1 g/l/h (in the case of the vacuum evaporation) to 0.5 g/l/h (in the case of biological treatment). The detoxified hydrolysate with biological treatment had the highest xylitol volumetric productivity in the maximum concentration of xylitol produced (>38 g/l xylitol, 90h) with a value of about 0.3 g/l/h, while the other detoxified hydrolysates result in xylitol productivity of around 0.1 g/l/h (maximum about 23 g/l xylitol, 254 h). The xylitol volumetric productivity reported with olive biomass by other authors range from 0.07 g/l/h for extracted olive pomace (Brás et al., 2014) to 0.1 g/l/h for olive tree pruning biomass (García et al., 2011) or 0.11 g/l/h for olive stone (one third of that achieved in this work).

Considering the achieved xylitol concentration, xylose consumption (Figure 7), and xylitol volumetric productivity (Figure 8), biological detoxification was found to be superior compared to the other detoxification methods studied. Furthermore, biological detoxification is chemical-free compared to other detoxification methods and therefore more environmentally friendly.

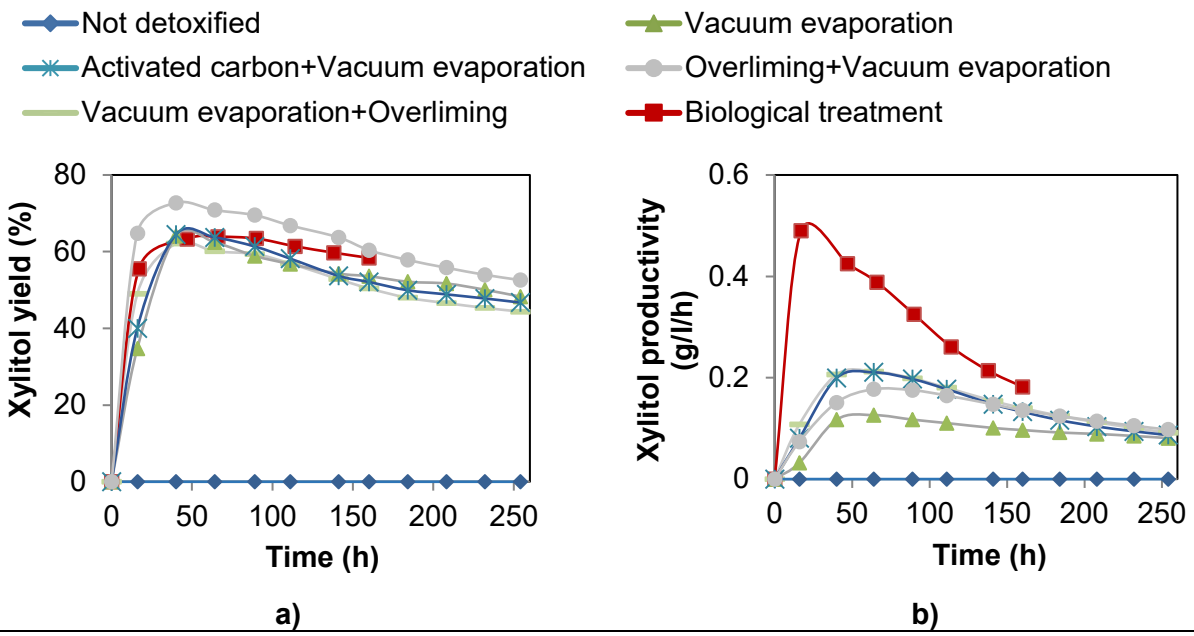


Figure 8. Evolution of yield (a) and volumetric productivity (b) of xylitol in the fermentation with *C. boidinii* of olive stone detoxified hydrolyzate using different detoxification methods (vacuum evaporation, overliming+ vacuum evaporation, activated carbon+ vacuum evaporation, vacuum evaporation+ overliming and biological treatment with *S. cerevisiae*)

3.5. Xylitol crystals

XRD, ATR FT-IF and TG analyses were performed on the fermented xylitol crystals, commercial xylitol crystals and analytical grade xylitol crystals. The obtained results were compared with each other and with data derived from public databases when it was relevant. XRD patterns (see Fig. S1 in supplementary material), ATR FT-IR spectra (Fig S2) and dTG curves (Fig S3) of fermented xylitol and commercial or standard xylitol matched very well confirming that the fermentative xylitol production resulted in xylitol crystals with high purity. Microscopic pictures also underline the great similarity between fermented and commercial xylitol (Fig. S4).

3.6. Suggested process scheme

In Figure 9 the material balance for the proposed process for the production of a liquid with antioxidant capacity and xylitol from OS using a two-stage treatment is shown. In the first stage, the olive stone is subjected to an aqueous extraction using a solid-liquid ratio of 50% at 130 °C for 90 min. After separation, 97.8% of solid was recovered and a liquid with 370 mg

GAE/100 g OS with good antioxidant properties (Lama-Muñoz et al., 2014) was obtained; this can be applied in various sectors such as food or cosmetic.

In the second stage, mainly hemicellulosic sugars were recovered by pretreatment with sulfuric acid (2 %w/v) with a solid-liquid ratio of 50% for 90 min at 130 °C and subsequent washing of the OS to improve final recovery of sugars (30 °C, 10 min, 220 rpm).

After separation, 68.8% of initial solid was recovered, mainly composed of cellulose and lignin, 30.2% and 54.9% respectively, which can be interesting for the recovery of lignin content and glucose. Glucose can be converted into bioethanol or into other products with high added value such as PHB, PLA or HMF and lignin into phenols, biopolymers or fibers.

The obtained hydrolysate is rich in xylose, which accounts for over 83% of total sugars (19.15 g/100 g OS), recovering over 70% of the original, which makes it very interesting for the production of xylitol (Rodrigues et al., 2003). Mixing the two liquid streams, that from acid treatment and that from washing, produces a solution with a xylose concentration above 60 g/l which is in the optimum range for xylitol production with *C. boidinii* according to the results of the design of experiments performed. The major inhibitor compound in the hydrolysate is acetic acid, accounting nearly 80% of all compounds measured (5.11 g/100 g OS), which has the greatest negative effect of the three factors studied. The hydrolysate was not fermentable so different detoxification methods were compared and the best results were obtained in the case of biological treatment with *S. cerevisiae*. The conditions of biological detoxification with *S. cerevisiae* are: initial cell concentration of 5 g/l, 30 °C, pH=5.5, 115h. With this treatment, acetic acid, levulinic acid, HMF and furfural were reduced to almost 0, glucose was completely consumed and the concentrations of arabinose and galactose were reduced, which is positive since it can reduce the cost of purification of xylitol. It is noteworthy that the consumed xylose was converted to xylitol with a yield near 80%, resulting 2.47 g/100 g OS. *S. cerevisiae* cells were separated after 115h and this detoxified hydrolysate was fermented with *C. boidinii* at 30 °C, pH=6 for 90 h with an initial cell concentration of 5 g/l. The total amount of xylitol produced was 11.96 g/100 g OS with more than 38 g/l (yield>62%), with practically total consumption of xylose and a very small amount of galactose remaining,

which will facilitate purification and crystallization of xylitol in the next step. The amount of xylitol produced by the proposed process was far superior to that obtained with olive tree pruning biomass and olive stone with other processes, 5.3 g/100 g and 9.2 g/100 g respectively (García et al., 2011, Saleh et al., 2014).

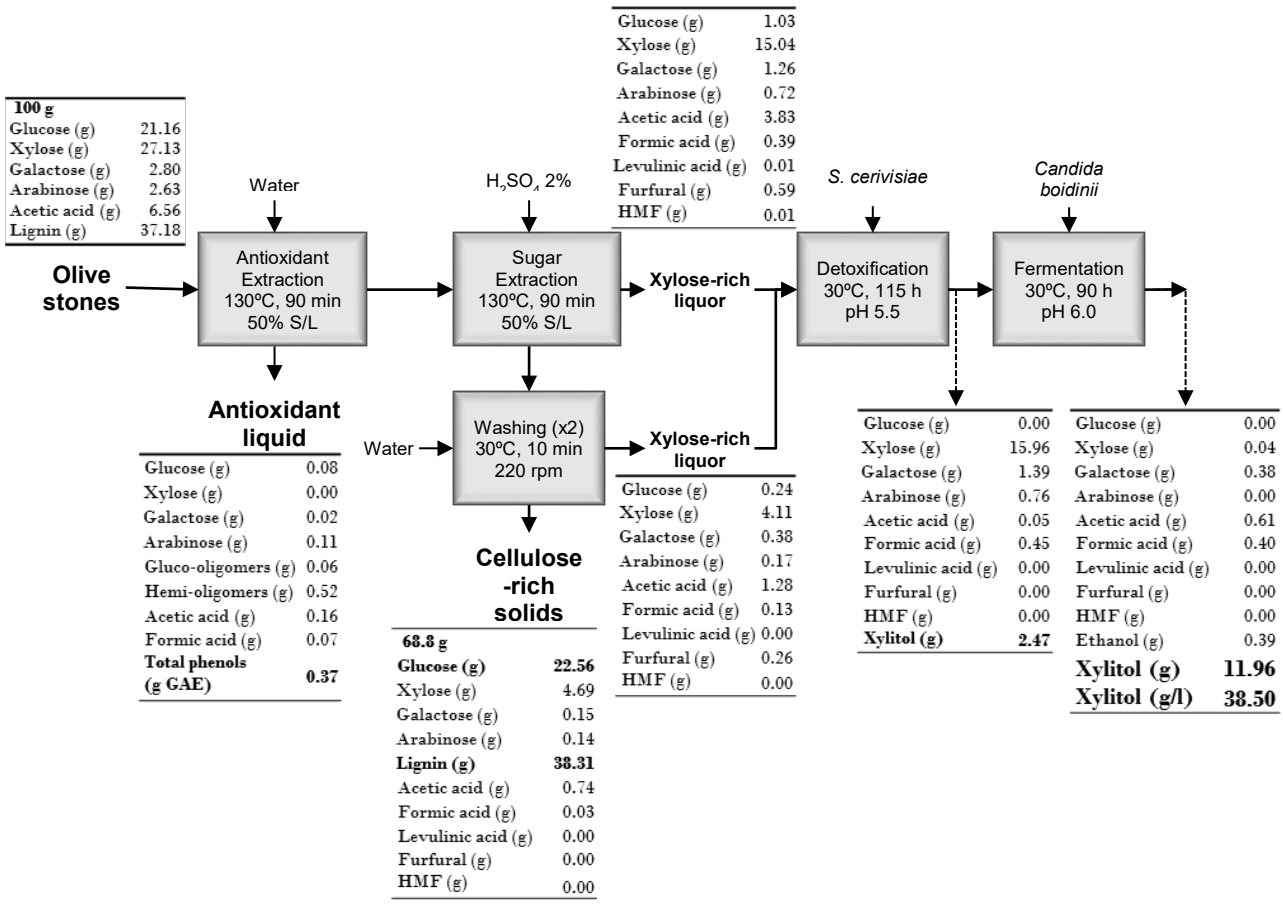


Figure 9. Material balance of the proposed two-stage process for the production of an antioxidant liquid and xylitol from olive stone

Conclusions

This paper proposes a process in two stages by which the olive stones can be valorised by obtaining a liquid with antioxidant capacity and xylitol, both products with high added value that could be used in various sectors such as food, pharmaceuticals and cosmetics. The resulting solid is an interesting by-product due to its high content of cellulose and lignin, and can be valorised in consecutive process steps. Statistical analysis and subsequent

optimization have determined that the optimum range of initial xylose concentration in the xylitol fermentation carried out with *C. boidinii* is between 55 and 76 g/l, if the initial concentration of cells and acetic acid is 5 g/l and 0 g/l, respectively. The combination of biological treatment with *Saccharomyces cerevisiae* as a detoxification method and subsequent fermentation with *C. boidinii* for the production of xylitol from olive stone hydrolysate resulted in a xylitol yield higher than 62%. Furthermore, this combination produces the total consumption of glucose, HMF, furfural, among other compounds, and almost total consumption of xylose and acetic acid (major compounds), which will facilitate the purification and crystallization steps of xylitol.

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