



Research article

Xylitol production by a *Wickerhamomyces anomalus* strain adapted for enhanced tolerance to sugarcane bagasse hemicellulosic hydrolysate with high content of fermentation inhibitors [☆]

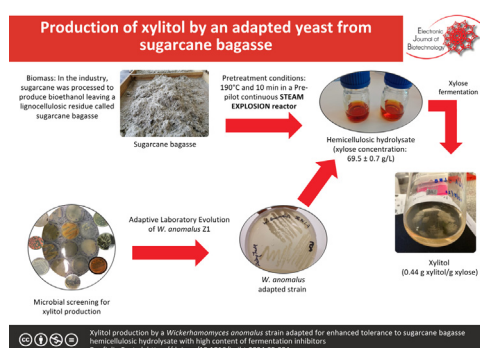


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GRAPHICAL ABSTRACT



Production of xylitol by an adapted yeast from sugarcane bagasse

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ABSTRACT

Background: Xylitol, a five-carbon polyalcohol, is used in the food and pharmaceutical industries and as a building block in the synthesis of high-value chemicals. It can be sustainably produced from renewable sources through xylose assimilating microbe fermentation.

Results: We screened microbial strains for xylitol production and identified *Wickerhamomyces anomalus* Z1 as a key xylitol producer. Utilizing lignocellulosic biomass hydrolysates for xylitol production poses challenges due to microbial sensitivity to inhibitors from biomass pre-treatment. In this study, an adaptive laboratory evolution (ALE) of *W. anomalus* Z1 was performed by culturing the yeast in a mineral medium supplemented with gradual increases of sugarcane bagasse hemicellulosic hydrolysate (SCHH) obtained by intensified steam explosion pretreatment. The performance of the adapted yeast, named *Wickerhamomyces anomalus* ALE, was assessed in comparison to the wild-type strain regarding its capacity to produce xylitol using SCHH. The evolved yeast reached a xylitol yield of 0.11 g xylitol/g xylose whereas the wild-type strain could not produce xylitol. Removing acetic acid from SCHH enhanced *W.*

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Wickerhamomyces anomalus Z1
Xylitol
Yeast

anomalus ALE performance, with optimal results at 75% hydrolyzed hemicellulose, yielding 0.44 g xylitol/g xylose and 13.41 g/L xylitol.

Conclusions: This study demonstrates the potential of *W. anomalus* ALE in successfully valorizing the hemicellulosic fraction of sugarcane bagasse for sustainable xylitol production.

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1. Introduction

Recently, attempts have been made to develop more efficient uses of agro-industrial residues to produce fuels and chemicals [1]. Specifically, within the use of pentoses present in lignocellulosic hydrolysates, the production of xylitol from these carbohydrates has been widely investigated [2,3]. Among the different lignocellulosic biomasses studied, sugarcane bagasse has with-drawn particular interest. After crushing and extracting the juice of the sugarcane (*Saccharum officinarum* L.), a fibrous lignocellulosic residue remains, the so-called sugarcane bagasse, a by-product of the sugar and first-generation bioethanol industries. The sugarcane bagasse has been utilized for countless purposes, from construction to bioethanol [4,5,6].

Xylitol is a five-carbon sugar alcohol ((2R,3R,4S)-pentane-1,2,3,4,5-pentol) and is present in several vegetables, fruits, and hard-wood trees at trace levels [7]. This white crystalline carbohydrate shares a sweetness like sucrose but with fewer calories and is commonly used as an artificial sweetener [8,9]. Due to its low caloric value, xylitol offers numerous health benefits and finds applications in the food, pharmaceutical, and dental industries due to its low gly-cemic index, insulin-independent metabolism, anticariogenic and prebiotic properties, and anabolic effects [7,8,10,11]. Also, it prevents demineralization of teeth and bones, otitis media, respiratory tract infections, inflammation, and cancer progression [12]. Additionally, it serves as a building block to high-value chemicals such as hydrox-yfuran, 1,2-propanediol, xylaric acid, glycols (propylene glycol and ethylene glycol), and lactic acid [8]. Recently, xylitol has gained prominence in materials science for its thermal properties and energy storage capabilities [13]. Due to these aspects, demand for xylitol is constantly increasing and is expected to expand in the global market from an estimated value of US\$ 1190.12 million in 2021 to US\$ 1475.87 million by 2030 [14]. As a result of its several features and applications, xylitol is classified as one of the major value-added chemicals that can be produced from biomass [15].

Xylitol can be produced chemically or by biotechnological pro-cesses. Industrial production of xylitol is an expensive, non-environmentally friendly, and energy-intensive process. It is car-ried out by a catalytic hydrogenation of purified xylose obtained by acid hydrolysis of lignocellulosic materials using metal as cata-lyst at severe conditions of pressure and temperature and requires a series of complicated purification procedures to separate the xyl-itol from other by-products formed during the production process [14]. Biotechnological methods to produce xylitol by microorgan-isms offer an alternative with several advantages since can be con-ducted under mild conditions that result in increased product yield and productivity and minimize the formation of by-products [14,16]. Additionally, the conversion of xylose to xylitol carried out by microorganisms using non-purified xylose, obtained from the hemicellulose-rich fraction of lignocellulosic biomass [17,18] has an added advantage in reducing production and purification costs [19].

Fermentation is a useful strategy in which filamentous fungi, bacteria, and yeast strains, are used for the synthesis of xylitol.

Xylitol is produced by these microorganisms as an intermediate in the xylose metabolic pathway [20]. Although filamentous fungi are robust biocatalysts due to their ability to produce enzymes, such as the xylanolytic complex, which can convert xylan-rich lig-nocellulosic residues into xylose, and the production of xylitol [21], these microorganisms have been little studied on its ability to pro-duce xylitol from lignocellulosic residues. Likewise, even though the use of bacterial cultures is advantageous due to their easy han-dling and rapid growth rate, there are few studies on the produc-tion of xylitol by bacterial strains [17,22]. To date, microbial fermentation is performed mainly by culturing yeasts using hemi-cellulosic hydrolysate as substrate due to improved yield and pro-ductivity compared to other microorganisms [22,23].

The production of xylitol from lignocellulosic feedstock requires pretreatments of biomass and hydrolysis steps for the solubilization of hemicellulosic sugars. Several pretreatment pro-cesses have been proposed for biomass deconstruction, among which, steam explosion is very efficient to disrupt the material structure [24]. The steam explosion pretreatment disrupts the lignocellulosic matrix, solubilizing the hemicelluloses into a range of monomers to oligomers of sugars. Adjusting the condi-tions of steam explosion -namely pressure and time of residence- also cellulose and lignin can be partially broken down, releasing inhibitory compounds that are generated from partial over-degradation of lignocellulose, partial breakdown of lignin components and degradation of sugars [25,26]. These inhibitors adversely affect the growth of microorganisms and thereby reduce the overall fermentation performance [3]. One of the main inhibitors is acetic acid formed by deacetylation of hemicellu-loses. This is a weak monocarboxylic acid that shows antimicro-bial effects mainly at low pH values. Acetate penetrates the cell in its undissociated form, but once inside the cytoplasm, it dissoci-ates, triggering high energy demand and an increase in cytoplas-mic pH values. This leads to inhibiting xylose metabolism even at relatively low concentrations [3].

Since one interesting feature of microorganisms is their ability to adapt rapidly to different environmental conditions [27], the inhibitor resistance of a selected fermenting microorganism can be improved through adaptive evolution [28]. Adaptive laboratory evolution (ALE) strategies allow for the metabolic engineering of microorganisms by combining genetic variation with the selection of beneficial mutations in an unbiased fashion [27]. The adaptation of microorganisms has been described as an efficient method for increasing the natural tolerance of microorganisms by pre-exposing them to non-lethal concentrations of inhibitors. As com-pared to un-adapted strains, the use of adapted strains can increase fermentation yields and productivity, even in high concentrations of inhibitors [14]. The implementation of adaptation strategies has been reported in previous studies for xylitol production [13,14,29].

Keeping the above points in view and improving xylitol produc-tion by microbial species, a collection of microorganisms was screened for their ability to produce xylitol. *Wickerhamomyces anomalus* Z1 was selected as an efficient producer of xylitol.

This strain was subjected to an ALE to increase its resistance to inhibitors present in the sugarcane bagasse hemicellulosic hydrolysate (SCHH). Both the wild type and the adapted strain *Wickerhamomyces anomalus* ALE were evaluated for their ability to produce xylitol using SCHH obtained by intensified steam explosion pretreatment. Finally, acetic acid was removed from two SCHH with different xylose concentrations, and xylitol production was studied using these fractions at 50, 75 and 100% w/w of each SCHH. In this way, it was possible to increase the yield of xylitol production using SCHH as substrate by *W. anomalus* ALE.

2. Materials and methods

2.1. Reagents

Xylose, xylitol, Yeast Nitrogen Base Without Amino Acids (YNB) and M9 broth were obtained from Sigma-Aldrich; yeast extract, peptone bacteriological, Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA) were obtained from Oxoid. All other chemicals were of analytical grade.

2.2. Feedstock

Sugar cane bagasse was provided by ALUR Alcoholes del Uruguay from the Bella Unión (Uruguay) bioethanol and refined sugar production plant. The feedstock was dried at 40°C by forced convection oven until 10% moisture and then milled to an average particle size of 1 cm.

2.3. Steam explosion pretreatment

The milled biomass was pretreated in a semi-continuous pre-pilot reactor. The equipment (Advance Bio Systems LLC, model S1401-D2011) has an approximate capacity of 10 kg h⁻¹ of raw biomass pretreatment. Three conditions were applied to the biomass to obtain the corresponding hemicellulosic hydrolysate. Condition 1: 190 °C of temperature and 10 min of residence, hereafter identified as 190.10. Condition 2: 200 °C and 5 min, from now on identified as 200.5; and the third condition of pretreatment was 200 °C and 10 min, henceforward identified as 200.10. After steam explosion pretreatment, the biomass sludge was pressed and filtered to separate the hemicellulosic liquid fraction from the solid fraction. The hemicellulosic liquid fraction was submitted to a post-hydrolysis process to hydrolyze the oligomers to monomers using 4% (w/w) sulfuric acid in autoclave at 121 °C for 15 min.

2.4. Hydrolysate detoxification

A liquid–liquid extraction detoxification process using ethyl acetate was applied to reduce the concentration of organic acids in the SCHH. The procedure involved the following steps: the SCHH pH was adjusted to pH >3 using a 2 M NaOH solution. A volume of SCHH and ethyl acetate in a 4:1 ratio was placed in a separation funnel. The funnel was vigorously shaken for a few seconds, allowing the phases to separate. The bottom phase corresponding to the SCHH fraction was removed, and the ethyl acetate was discarded. This process was repeated four times. Finally, the SCHH fraction was vacuum evaporated (BUCHI Series R-100 Rotavapor) to completely remove the ethyl acetate. The acetic acid content in the SCHH was analyzed before and after the detoxification process using HPLC. To be used as fermentation medium, the hydrolysates had the pH adjusted with CaCO₃ to 5.5 and filtered using a Thermo Scientific™ Nalgene Filter (Waltham, United States) with polyethersulfone membrane and pore size of 0.2 mm for sterilization.

2.5. Microbial strains

2.5.1. Microbial strains used in screening for xylitol production

Twenty-six bacteria, ten filamentous fungi, and eight yeasts isolated from different environmental sources [30,31,32,33,34,35,36] were screened to produce xylitol. Bacterial and yeast strains were maintained as frozen cultures in 17% sterile glycerol at –70 °C. In the case of filamentous fungi, pieces of agar with grown colonies were transferred to sterile vials with distilled water and maintained at 5°C. Microbial strains were deposited in the microbial collection of Laboratorio de Biocatálisis y Biotransformaciones de la Facultad de Química, Udelar.

The microbial strains investigated were *Enterobacter agglomerans* C8, *Pantoea* sp. C13, *Micrococcus luteus* C20, *Bacillus subtilis* C28, *Leuconostoc* sp., *Pseudomonas* sp. EB, *Bacillus megaterium* CM15, *Pseudomonas* sp. Eix1aTSA, *Roseomonas* sp. CM14, *Stenotrophomonas* sp., *Bacillus* sp., *Pseudomonas* sp. E, *Citrobacter freundii* A1, *Klebsiella oxytoca* S2, *Enterobacter* sp., *Enterobacter agglomerans*, *Klebsiella pneumoniae* A, *Citrobacter freundii* S2, *Citrobacter* sp., *Enterobacter agglomerans* EL1, *Rhodococcus* sp. T4A, *Bacillus* sp. 12–22, *Citrobacter* sp. M3U, *Exiguobacterium* sp. M3S, *Acinetobacter* sp. M3X1, *Proteus* sp. M1E, *Gymnopilus spectabilis* 7423, *Dichostereum sordulentum* 7454, *Aspergillus terreus* BFQU121, *Trichoderma* sp. H3, *Trichoderma* sp. Y5, *Aspergillus* sp. Y6, *Rhizopus* sp., *Mucor* sp. Y2, *Penicillium* sp. VA8, *Fusarium* sp. IBH10, *Pichia* sp. PDA, *Rhodotorula* sp. Ro, *Aureobasidium pullulans* CQA, *W. anomalus* Z1, *Rhodotorula glutinis* H93, *Rhodotorula mucilaginosa* H2, *Pseudozyma* sp. 97–87 and *Aureobasidium* sp. 99–86.

2.5.2. ALE of *W. anomalus* Z1 for SCHH tolerance

The adaptive laboratory evolution approach was used aiming to increase SCHH tolerance of *W. anomalus* Z1. The procedure involved the following steps: a single colony from a fresh culture of the *W. anomalus* Z1 was inoculated into a 100 mL Erlenmeyer flask containing 40 mL of YPX broth (10 g/L yeast extract, 20 g/L peptone, and 15 g/L xylose) and incubated at 28 °C at 150 rpm on an orbital shaker (IKA KS4000 ic) for 48 h. After that, 1 mL from the primary inoculation was transferred to 100 mL Erlenmeyer flasks containing 40 mL of YP medium (10 g/L yeast extract and 20 g/L peptone) and the SCHH (condition 200.10). Sequential transfers of the inoculum (1 mL) were performed into media with increasing concentrations of SCHH (condition 200.10) (5, 10, 25, 50, 75, and 100%), allowing the strains to adapt to the physiological conditions of the medium. Cell culture growth PDA plates were used to monitor each step during the successive inoculation of yeast until a significant increase in cell concentration was observed.

2.6. Screening of xylitol production by xylose-assimilating microorganisms

In the general procedure for xylitol production, fresh plates of bacterial strains were streaked from frozen stock onto tryptic soy agar (TSA). A single colony was used to inoculate 5 mL of M9 broth (1 g/L ammonium chloride, 6 g/L disodium hydrogen phosphate, 3 g/L potassium dihydrogen phosphate, and 0.5 g/L sodium chloride) supplemented with 1% xylose. The cultures were incubated at 28 °C and 150 rpm on orbital shaker for 48 h. Then, 100 µL of each culture was used to inoculate 5 mL of YPX broth (20 g/L peptone, 10 g/L yeast extract, and 30 g/L xylose) in a 25 mL Erlenmeyer flask. The temperature and agitation speed were maintained at 28 °C and 150 rpm (orbital shaker). Samples were collected at 48 h for quantification of xylose and xylitol. The same procedures were followed for xylitol production with filamentous fungi strains, using three pieces of 1 cm diameter mycelial growth to inoculate 20 mL

of YPX broth in a 100 mL Erlenmeyer flask, incubated at 28 °C and 150 rpm for 144 h. For yeast strains, fresh plates were streaked from the frozen stock onto potato dextrose agar (PDA). A single colony was used to inoculate 40 mL of YP broth supplemented with 1.5% xylose (10 g/L yeast extract, 20 g/L peptone, and 15 g/L xylose) in a 100 mL Erlenmeyer flask, which was then incubated at 30 °C and 150 rpm for 24 h. Afterward, the cells were recovered by centrifugation (Thermo Scientific Sorvall RC6 Plus™ Superspeed Centrifuge) and washed twice with 0.9% NaCl solution and inoculated into another 40 mL of YPX broth. Samples were collected at 48 h for quantification.

2.7. Fermentations to evaluate the performance of xylitol production by *W. anomalus* Z1 and *W. anomalus* ALE

Fermentation experiments using media with pure xylose and SCHH detoxified were performed with the aim of comparing the ability of *W. anomalus* Z1 and *W. anomalus* ALE to produce xylitol. To accomplish this objective, colonies isolated from each strain were taken and inoculated into 250 mL Erlenmeyer flasks containing 40 mL of liquid YPX broth. These flasks were incubated at 30 °C, 150 rpm for 24 h. Afterward, the cells were recovered by centrifugation and washed twice with 0.9% NaCl solution. Biomass was transferred to 25 mL Erlenmeyer flasks containing 10 mL of YNB medium supplemented with 25 g/L xylose, as well as to 25 mL Erlenmeyer flasks containing 10 mL of detoxified SCHH (condition 200-10) with previous pH adjustment to 5.5 with CaCO₃ and sterilized. These flasks were incubated at 30 °C and 150 rpm. Samples were regularly collected at the start and every 24 h up to 96 h for measurement of xylose and xylitol concentrations and cell dry weight. Assays were performed in duplicate.

2.8. Fermentation of detoxified hydrolysates by *W. anomalus* ALE

The fermentation of detoxified and post-hydrolyzed SCHH by *W. anomalus* ALE was carried out in the same way as described in 2.7 using 50, 75 and 100% of each SCHH obtained in 2.3 and detoxified as described in 2.4 in YNB medium. Samples were regularly collected at the start, 24 h and 48 h for measurement of xylose and xylitol concentrations and cell dry weight. Assays were performed in duplicate.

2.9. Analytical methods

The composition of the sugarcane bagasse hydrolysates was determined by high-performance liquid chromatography using a Dionex Ultimate 3000 UHPLC. Acetic acid, formic acid, 5-hydroxymethylfurfural (5-HMF) and furfural were quantified with a Bio-Rad Aminex[®] column HPX-87H (300 mm x 7.8 mm) at 60 °C using refractive index detector and a UV-Vis at 254 nm for 5-HMF and furfural, 5 mM H₂SO₄ as mobile phase at 0.6 mL/min. Carbohydrates were determined using a Bio-Rad Aminex[®] column HPX-87P at 65 °C using refractive index detector, deionized water as mobile phase at 0.6 mL/min. Total phenolics were quantified by colorimetric method using gallic acid as standard, adapted from [37]. A total of 200 µL of sample were mixed with 100 µL Folin-Ciocalteu reagent, 900 µL of water, and 800 µL of Na₂CO₃ 7.5% m/v, heated at 60 °C for 5 min and allowed to cool in darkness at room temperature. The absorbance was measured at 760 nm using a Mettler Toledo UV-Vis Excellence spectrophotometer.

During fermentation assays, xylose, xylitol, and acetic acid concentrations were determined by high-performance liquid chromatography (HPLC) using a Shimadzu LC – 20AT equipped with a refractive index detector (Shimadzu RID10-AT) using a Supelcogel C610H (30 cm × 4.6 mm) column at 55°C, eluted at 0.5 mL/min, 0.005 N sulfuric acid. Fermentation samples for HPLC analysis were

centrifuged at 10,000 rpm, 5 min, 4–5 °C (Sorvall™ Legend™ Micro 17R Microcentrifuge). Supernatants were kept in the freezer at –20 °C until analysis. Cell concentration was determined by dry weight. For analysis, 1 mL of sample with cells was centrifuged (10,000 rpm, 5 min, 4–5 °C) and the pellet dried to constant weight at 100°C. The cell concentration was calculated as the ratio between the mass of dried biomass and the filtered wet sample. All analytical determinations were performed in triplicate, and average results are shown.

2.10. Fermentation parameters

Xylitol yield ($Y_{P/S}$, g/g) was calculated as the ratio between xylitol produced and xylose consumed, while the xylitol productivity (Q_P , g/L.h) was determined as the ratio between the xylitol concentration (g/L) and the fermentation time (h), respectively.

3. Results and discussion

3.1. Xylitol production by xylose-assimilating microorganisms

Microorganisms utilize isomerase, oxidative, or oxidoreductase metabolic pathways to metabolize xylose [38]. On the oxidoreductase pathway, xylose is reduced to xylitol that is secreted out of the cell or otherwise, oxidized to xylulose, which is phosphorylated to xylulose-5-phosphate and integrated into the pentose phosphate pathway (PPP). The secretion or oxidation of xylitol depends on the availability of cofactors [19]. While the oxidoreductase pathway is common in eukaryotes such as yeast and filamentous fungi, research has also demonstrated that some bacteria metabolize xylose via this pathway [39].

The world market's increasing demand for xylitol has generated great interest in finding new microbial strains that produce xylitol with better efficiency [22]. In the present study, twenty-six bacteria, ten filamentous fungi, and eight yeasts isolated from environmental sources were screened to produce xylitol. Of them, twelve strains were able to produce xylitol when pure xylose was used as the carbon source (Table 1). The xylitol production was low under the conditions evaluated, with the yeast strains showing the best xylitol production. It agrees with previous reports that show the higher capacity of yeast strains in the production of xylitol compared to other microorganisms [19,23,40]. Among the strains screened, the endophytic yeast isolated from *Cucurbita maxima* and identified as *W. anomalus* Z1 [35] produced the highest amount of xylitol and the best productivity. A recent study by Carneiro et al. [41] found that *W. anomalus* 740 produced a high yield (0.83 g xylitol/g xylose) and good xylitol production (24.75 g/L) in sugarcane bagasse hydrolysate and presents a high tolerance to acetic acid.

Table 1
Xylitol production by screened microorganism strains in liquid cultures with xylose as single carbon source.

Microbial strains	Xylitol (g/L)	$Y_{P/S}$ (g/g)	Q_P (g/Lh)
<i>Pantoea</i> sp. PRII45C13	1.70	0.40	0.04
<i>Enterobacter agglomerans</i> PRII45C8	1.00	0.11	0.02
<i>Citrobacter</i> sp.	0.70	0.05	0.01
<i>Bacillus megaterium</i> CM15	0.40	0.05	0.01
<i>Aspergillus</i> sp. Y6	0.72	0.04	0.01
<i>Trichoderma</i> sp. H3	0.34	0.03	0.01
<i>Mucor</i> sp. Y2	0.50	0.02	0.01
<i>Penicillium</i> sp. VA8	0.97	0.03	0.02
<i>Rhodotorula</i> sp. Ro	2.81	0.07	0.04
<i>W. anomalus</i> Z1	14.46	0.29	0.20
<i>Pichia</i> sp. PDA	11.65	0.25	0.16
<i>Rhodotorula</i> sp. EL4	4.50	0.11	0.06

W. anomalus is an ascomycetous yeast (formerly named *Pichia anomala* and *Hansenula anomala*) [42]. This yeast has special characteristics that make it a potential biocatalyst for obtaining xylitol. It is a robust microorganism that could grow under stressful environmental conditions [43], such as extremes of pH, low water activity, and anaerobic conditions [42], and has antimicrobial properties [44]. Regarding food safety aspects, this yeast is classified at biosafety level 1 and is considered safe for healthy individuals [44]. Currently, it has QPS (Qualified Presumption of Safety) proposed by the European Food Safety Authority of the EU (EFSA), which gives it benefits in terms of public perspectives of food biotechnology [45].

3.2. Characterization of the SCHH

The composition of the liquid fraction obtained after the steam explosion was analyzed for the three conditions applied to the sugarcane bagasse. The results are shown in Table 2. The effect of steam explosion on xylose concentration in the hydrolysate was investigated to optimize pretreatment for xylose recovery from biomass. In general, the concentrations of the compounds are similar to different studies in comparable conditions [46,47,48]. As can be seen, the original hydrolysate of the most severe condition (i.e., 200.10, 200 °C and 10 min of residence) had a higher concentration of xylose. However, the presence of cellobiose in all three conditions, and with higher concentration in the other two conditions (190 °C and 10 min of residence, and 200 °C and 5 min of residence) indicated that oligomers of sugars were present. Therefore, a post-hydrolysis of the hemicellulosic hydrolysate to hydrolyze the oligomers and increase the concentration of xylose was performed. Concentrations of the compounds after post-hydrolysis are shown in Table 2. In the post-hydrolyzed SCHH, the condition 190.10 increased six times the concentration of xylose, being the condition with the highest amount of it after hydrolysis. On the other hand, the concentration of xylose in the condition 200.10 was only doubled. This effect is explained by the fact that in the most severe condition, the hemicelluloses are more degraded, and therefore, less xylose oligomers are present in the hydrolysate. Also, the acetic acid concentration in the original hydrolysates was higher in the 200.10 min condition, whereas this is reversed after the post-hydrolysis, since the xylose oligomers –derived from the xylan present in the original sugarcane bagasse– had acetyl groups that were hydrolyzed. The higher concentration of acetic acid is a negative side effect of obtaining higher concentration of xylose since this acid is toxic to yeasts. It is worth noting that the concentration of the phenolic compounds, that may be detrimental to the yeast in the next steps, was lower after post-

hydrolysis for all the conditions. This positive outcome is due to the acidic precipitation of the phenolic compounds [49].

The toxicity of acetic acid formed by deacetylation of hemicelluloses on yeasts is well known in the literature [3,50]. In this sense, Vajzovic et al. [51] reported that in the presence of 5–20 g/L, acetic acid negatively affected xylitol production from xylose by *Rhodotorula mucilaginosa* PTD3. Also, Bellido et al. [52] reported the inhibition of ethanol production by *Pichia stipitis* at an acetic acid concentration of 3.5 g/L. Likewise, Björling and Lindman [53] described the complete inhibition of ethanol production by *Pichia stipitis* in a synthetic medium containing 3.9 g/L acetic acid at pH 4. Other authors described that xylose consumption rates in yeasts were affected by the presence of acetic acid at concentrations of 2.4–3 g/L [54,55]. Therefore, we carried out a detoxification process to reduce the concentration of acetic acid in the hydrolysates to obtain a suitable medium for fermentation. The concentration of acetic acid in the hydrolysates detoxified was approximately one-tenth of the concentration of acetic acid before the detoxification process.

3.3. ALE of *W. anomalus* Z1 to enhance its fermentability

The availability of a robust fermenting microorganism with high tolerance to inhibitors that are produced during biomass pre-treatment is critical for the sustainable and economical production of bio-based chemicals produced from lignocellulosic feedstocks [28]. Adaptive Laboratory Evolution (ALE) of microorganisms is a strategy to improve microbial performance during fermentation as it promotes the adaptation of cells to specific stress conditions, being a useful tool to develop the ability of strains to grow in media containing lignocellulosic inhibitors such as furan derivatives, phenolic compounds, and organic acids [13]. In this regard, it has been reported that yeasts that have successfully adapted to sugarcane bagasse hydrolysates exhibit enhanced resistance to inhibitors compared to the parental strain [56,57]. *W. anomalus* Z1 was selected as the parental strain for ALE process. This process was performed by sequential transfer of the yeast culture to a new medium containing an increasing concentration of SCHH (condition 200.10). The performance of xylitol production by *W. anomalus* ALE adapted was compared with the parental strain in a minimal media with pure xylose and in a medium with detoxified SCHH (condition 200.10). Fig. 1a shows that when pure xylose was used in the minimal media, xylitol production was similar with both strains. The maximum xylitol concentration obtained was 11.77 g/L corresponding to 0.50 g xylitol/g xylose with wild-type yeast. Only the adapted strain produced xylitol

Table 2
Chemical composition of Sugarcane Bagasse Hemicellulosic Hydrolysates (SHCC) obtained by steam explosion pretreatment.

Compound	Concentration in the hydrolysate (g/L)					
	Original Hydrolysate			Post-hydrolysis		
	Condition 190.10	Condition 200.5	Condition 200.10	Condition 190.10	Condition 200.5	Condition 200.10
Cellobiose	9.4 ± 0.2	7.7 ± 0.4	5.4 ± 0.2	Not detectable	Not detectable	Not detectable
Glucose	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	6.2 ± 0.3	6.3 ± 0.6	7.0 ± 0.6
Xylose	11.3 ± 0.3	10.4 ± 0.3	14.5 ± 0.2	69.5 ± 0.7	58.5 ± 0.5	33.4 ± 0.5
Arabinose	1.5 ± 0.1	1.3 ± 0.2	3.1 ± 0.1	3.0 ± 0.1	3.1 ± 0.3	1.5 ± 0.1
Acetic acid	6.9 ± 0.1	4.1 ± 0.1	9.6 ± 0.2	16.3 ± 0.2	14.2 ± 0.1	13.1 ± 0.1
Formic acid	4.5 ± 0.2	2.2 ± 0.3	4.3 ± 0.2	1.6 ± 0.1	1.1 ± 0.3	2.2 ± 0.2
5-HMF	0.2 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Furfural	0.9 ± 0.1	0.9 ± 0.1	1.9 ± 0.2	4.3 ± 0.2	4.2 ± 0.2	4.5 ± 0.4
Phenolic compounds	7.9 ± 0.2	6.0 ± 0.2	8.0 ± 0.2	3.0 ± 0.2	2.7 ± 0.2	3.6 ± 0.1

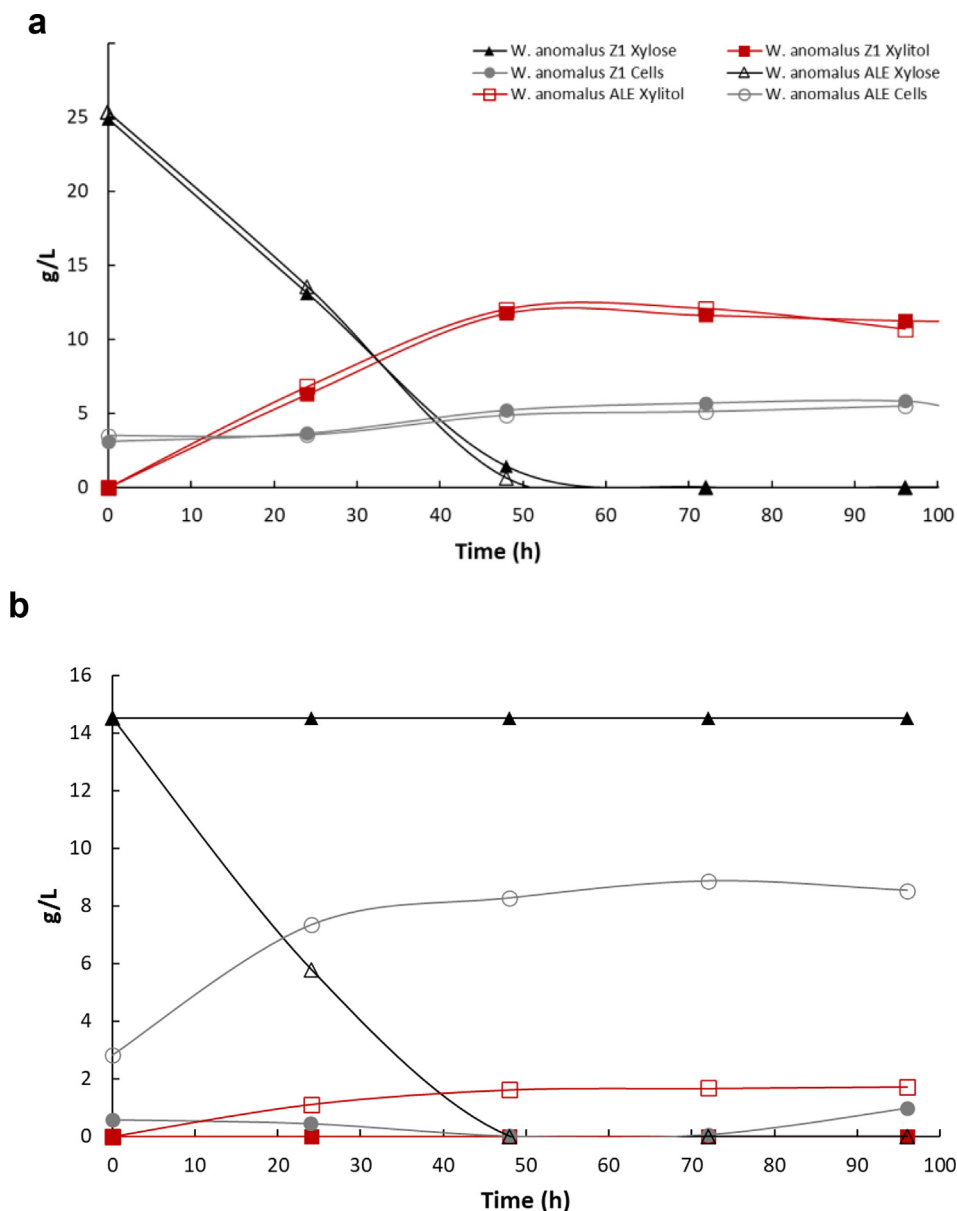


Fig. 1. Batch cultures of non-adapted *W. anomalous* Z1 and adapted *W. anomalous* ALE. (a) Minimal media with pure xylose and (b) media with 100% SCHH detoxicated. The results represent the average of the duplicates.

when the fermentation was carried out in the medium 100% w/w of detoxified SCHH with a yield of 0.11 g xylitol/g xylose (Fig. 1b).

Although *W. anomalous* ALE produced xylitol in a culture medium containing 100% w/w of detoxified SCHH, the yield in xylitol production was lower than that obtained in the minimal media with pure xylose. This result is probably due to the low xylose concentration in the media (Fig. 1b) since the initial xylose concentration is a key factor influencing xylitol production [8,58].

3.4. Fermentation of detoxified and post-hydrolyzed sugarcane bagasse hydrolysates by *W. anomalous* ALE

As presented in Fig. 2a, *W. anomalous* ALE completely consumed xylose at 48 h and produced xylitol in concentrations of 11.96 and 6.26 g/L, with yields of 0.36 and 0.25 g xylitol/g xylose in the media with 50% w/w detoxified and post-hydrolyzed SCHH 190.10 and 200.5 respectively. In 75% detoxified and post-

hydrolyzed SCHH media, total xylose consumption was not observed, however, a better yield of 0.44 g xylitol/g xylose was obtained in the medium with the detoxified and post-hydrolyzed SCHH 190.10 and the highest xylitol concentration of 13.41 g/L (Fig. 2b). Comparison of xylitol production results with those obtained by other authors is difficult because SCHH may contain different amounts and types of inhibitors, and microorganisms may have various levels of tolerance to these inhibitors. Nevertheless, an attempt was made to compare xylitol production achieved in the current study with other reports using SCHH under different conditions (Table 3). The range of concentrations and yields of xylitol obtained in these studies varies depending on inhibitor composition, detoxification method, fermentation conditions, and biocatalyst. Mostly, *Candida* species are the best producers of xylitol compared to other microorganisms. However, *Candida* species are considered opportunistic pathogens and their use in biotechnological processes has been

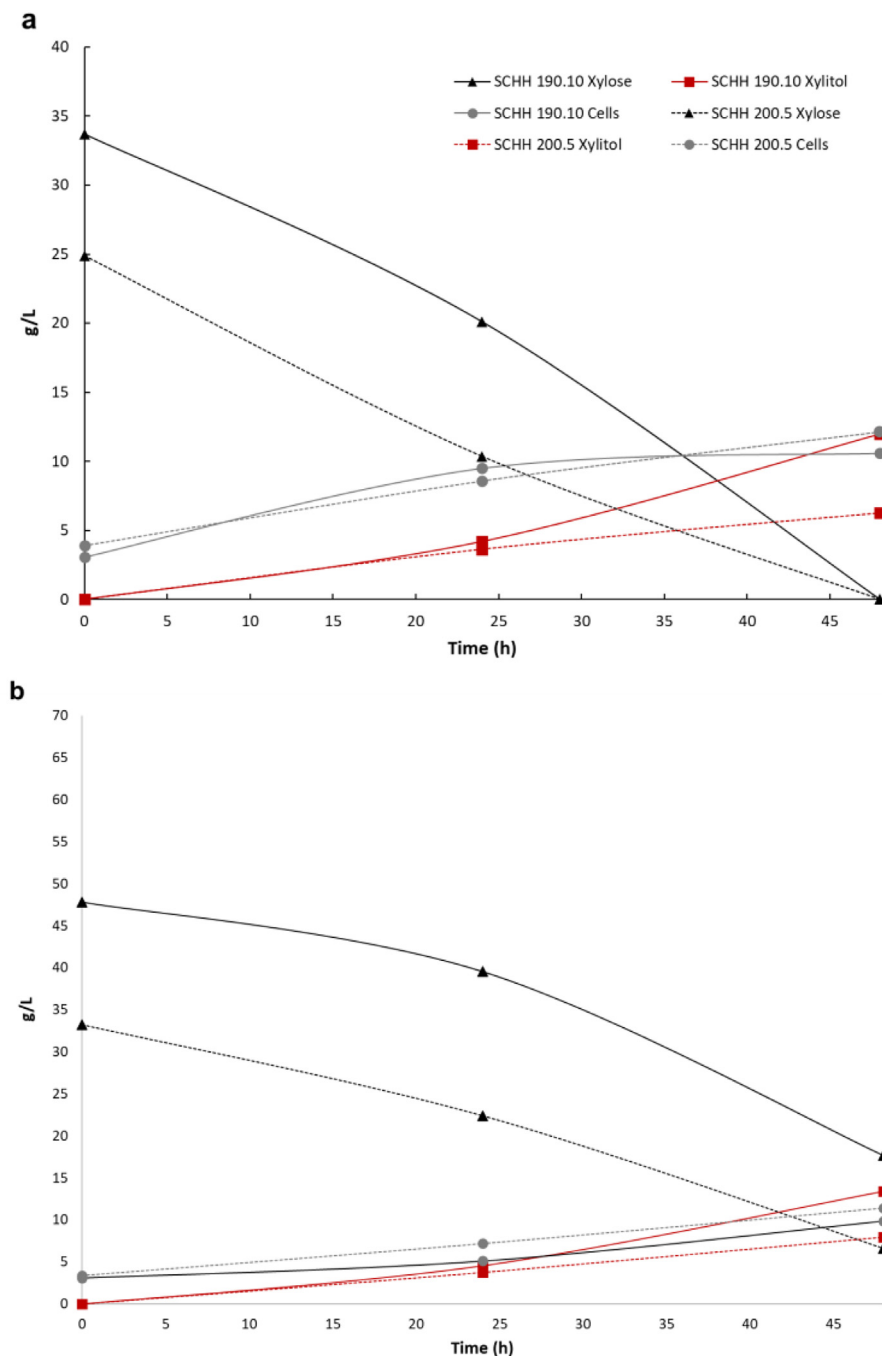


Fig. 2. Xylitol production profile by *W. anomalus* ALE in detoxified and post-hydrolyzed SCHH 190.10 and 200.5 at (a) 50% in minimal media; (b) 75% in minimal media and (c) 100%. The results represent the average of the duplicates.

questioned due to the lack of a GRAS status (generally recognized as safe) [17,41]. *W. anomalus* strains have been studied extensively for their biotechnological purposes in various fields, including medical applications [59], food and beverage processing [60,61], environmental bioremediation [62], and biofuel production [63]. Therefore, *W. anomalus* Z1 is a highly promising candidate for use in xylitol production.

Finally, *W. anomalus* ALE in non-diluted detoxified and post-hydrolyzed SCHH produced xylitol in a minor concentration when the SCHH 200.5 was used (Fig. 2c). Also, in 100% w/w of SCHH 190.10, *W. anomalus* ALE was able to produce 0.59 g/L xylitol, with a yield of 0.97 g xylitol/g xylose (Fig. 2c). This result agrees with

Morais et al. [3] who reported that the inhibitory effects of hydrolysate severely affect yeast growth, and carbon was directed almost exclusively to xylitol production when using *Candida tropicalis* JA2 to produce xylitol in 100% hydrolysate from sugarcane biomass. Also, Vallejos et al. [67] showed the strong inhibitory influence of acetic acid and phenolic compounds when undetoxified SCHH was used to produce xylitol by *C. tropicalis*. Despite the low consumption of xylose by *W. anomalus* ALE under these conditions, it is observed that the yeast continues to be viable and can grow (Fig. 2c). This result encourages us to continue with the study of the ability to produce xylitol with adapted yeast using lignocellulosic residues.

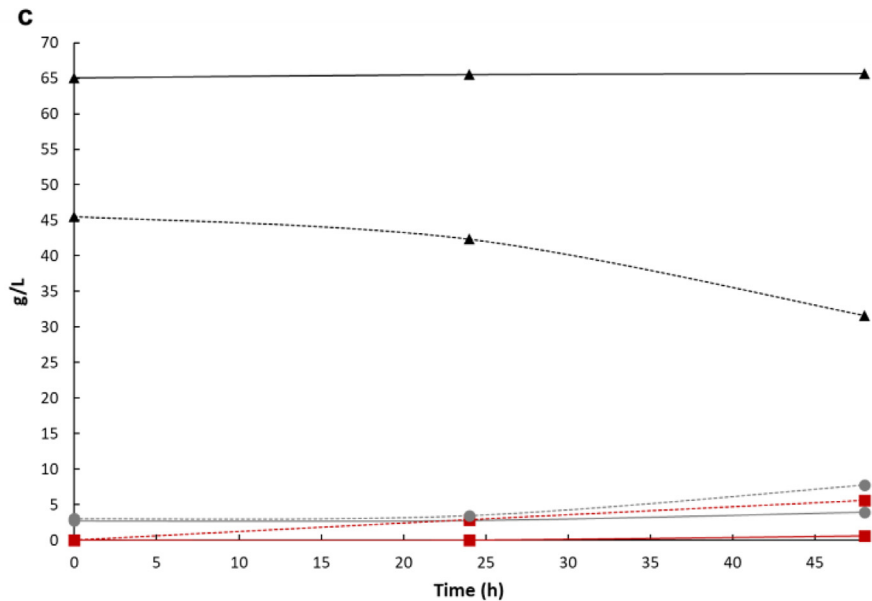


Fig. 2 (continued)

Table 3
Comparison data of studies on xylitol production from SCHH in batch mode.

Pretreatment	Microorganism	Media	Time of fermentation (h)	Initial xylose (g/L)	Xylitol (g/L)	$Y_{p/s}$ (g/g)	Q_p (g/Lh)	Reference
Steam explosion followed by acid hydrolysis	<i>D. hansenii</i>	SCHH detoxified by activated charcoal and supplemented with malt extract, yeast extract, and mycological peptone	60	20	14	0.69	0.24	[64]
Steam explosion followed by acid hydrolysis	<i>C. tropicalis</i> JA2	40% SCHH supplemented with YNB and urea	39	220	109.5	0.86	2.80	[3]
Dilute-base hydrolysis and xylanase hydrolysis	<i>C. tropicalis</i> MTCC 184	SCHH supplemented with yeast extract, malt extract and peptone	40	42.6	34.5	0.83	0.86	[65]
Dilute-acid hydrolysis	<i>C. guilliermondii</i> FTII 20037	SCHH detoxified with CaO, H_3PO_4 and activated charcoal. Supplemented with $(NH_4)_2SO_4$, $CaCl_2 \cdot 2H_2O$ and rice bran extract	132	60	41.8	0.66	0.29	[66]
Autohydrolysis followed by acid hydrolysis	<i>C. tropicalis</i>	SCHH detoxified with $Ca(OH)_2$, activated charcoal and anion exchange resin	120	104.1	32	0.46	0.27	[67]
Hydrothermal pre-treatment done by Nova Pangaea Technologies.	<i>P. fermentans</i> E015	Adjusted the pH of SCHH to 7.0 to extenuated toxic effect of acetic acid Supplemented with $(NH_4)_2SO_4$, $KH_2(PO_4)$ and yeast extract	168	150	79	0.54	0.47	[1]
Dilute-acid hydrolysis	<i>C. guilliermondii</i> FTI 20037	SCHH detoxified with CaO and activated charcoal. Supplemented with $(NH_4)_2SO_4$, $CaCl_2 \cdot 2H_2O$ and rice bran extract	120	60	36.3	0.64	0.76	[68]
Dilute-acid hydrolysis	<i>C. xylosilytica</i> UFMG-CM-Y309	SCHH detoxified with CaO and activated charcoal. Supplemented with $(NH_4)_2SO_4$, rice bran extract and yeast extract	72	40	14.06	0.63	0.20	[69]
Steam explosion followed by acid hydrolysis	<i>W. anomalus</i> 740	70–80% SCHH supplemented with xylose (12 g/L to reach 40 g/L) and glucose (2 g/L to reach 10 g/L)	40	30	24.75	0.83	0.10	[41]
Steam explosion followed by acid hydrolysis	<i>W. anomalus</i> ALE	75% SCHH detoxified by acetic acid extraction and supplemented with YNB	48	48	13.41	0.44	0.28	Present work

4. Conclusions

Several microorganisms were screened and tested for their ability to produce xylitol from xylose as a carbon source. Among them,

W. anomalus, a yeast classified as biosafety level 1, produced the highest amount of xylitol and the best yield. Using a laboratory adaptive evolution strategy, the *W. anomalus* ALE strain was obtained, which was able to grow in media containing lignocellu-

losic inhibitors produced after pretreatment of biomass with steam explosion, such as furan derivatives, phenolic compounds, and organic acids. Furthermore, this adapted yeast was able to produce xylitol under these culture conditions.

The conditions of the steam explosion pretreatment of sugarcane bagasse were determined in which the highest concentration of xylose in the hemicellulosic fraction was obtained. These fractions were used in different concentrations in the culture media, and it was determined that *W. anomalus* ALE produced the best yield and the highest concentration of xylitol in 75% detoxified SCHH media. Additionally, it was interesting to determine that this yeast can grow and produce xylitol in a medium containing 100% SCHH. These results encourage us to continue studying xylitol production using the adapted yeast to determine the optimal conditions for polyol production and its subsequent scale-up. In this way, it would be possible to obtain an environmentally friendly xylitol production process, which can be integrated into the production of ethanol as a biorefinery strategy.

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Conflict of interest

The authors of the manuscript titled “Production of xylitol by a strain of *Wickerhamomyces anomalus* adapted for greater tolerance to the hemicellulosic hydrolyzate of sugarcane bagasse with a high content of fermentation inhibitors” are Fernando Bonfiglio, Matías Cagno, Lucía Nuñez, Rossina Castro, Emiliana Botto, and Paula Rodríguez Bonnacarrere declares that they have no conflicts of interest.

CRediT authorship contribution statement

Fernando Bonfiglio: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Matías Cagno:** Methodology, Formal analysis. **Lucía Nuñez:** Methodology, Formal analysis. **Rossina Castro:** Methodology, Formal analysis. **Emiliana Botto:** Methodology, Investigation, Formal analysis. **Paula Rodríguez:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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Supplementary material

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