

Research article

Multi-feedstock biorefinery concept: Valorization of winery wastes by engineered yeast

Sara L. Baptista^{a,b}, Aloia Romaní^c, Joana T. Cunha^{a,b}, Lucília Domingues^{a,b,*}^a CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal^b LABBELS - Associate Laboratory, Braga, Guimarães, Portugal^c Department of Chemical Engineering, Faculty of Science, University of Vigo (Campus Ourense), As Lagoas, 32004, Ourense, Spain

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ABSTRACT

The wine industry produces significant amounts of by-products and residues that are not properly managed, posing an environmental problem. Grape must surplus, vine shoots, and wine lees have the potential to be used as renewable resources for the production of energy and chemicals. Metabolic engineering efforts have established *Saccharomyces cerevisiae* as an efficient microbial cell factory for biorefineries. Current biorefineries designed for producing multiple products often rely on just one feedstock, but the bioeconomy would clearly benefit if these biorefineries could efficiently convert multiple feedstocks. Moreover, to reduce the environmental impact of fossil fuel consumption and maximize production economics, a biorefinery should be capable to supplement the manufacture of biofuel with the production of high-value products. This study proposes an integrated approach for the valorization of diverse wastes resulting from winemaking processes through the biosynthesis of xylitol and ethanol. Using genetically modified *S. cerevisiae* strains, the xylose-rich hemicellulosic fraction of hydrothermally pretreated vine shoots was converted into xylitol, and the cellulosic fraction was used to produce bioethanol. In addition, grape must, enriched in sugars, was efficiently used as a low-cost source for yeast propagation. The production of xylitol was optimized, in a Simultaneous Saccharification and Fermentation process configuration, by adjusting the inoculum size and enzyme loading. Furthermore, a yeast strain displaying cellulases in the cell surface was applied for the production of bioethanol from the glucan-rich cellulosic. With the addition of grape must and/or wine lees, high ethanol concentrations were reached, which are crucial for the economic feasibility of distillation. This integrated multi-feedstock valorization provides a synergistic alternative for converting a range of winery wastes and by-products into biofuel and an added-value chemical while decreasing waste released to the environment.

1. Introduction

The wine industry is culturally and commercially important, constituting a large sector of global agriculture (FAO and OIV, 2016). Grapes are one of the most commonly cultivated fruit crops worldwide, with an estimated vineyard area of 7.3 million hectares in 2021 (OIV, 2022). Spain (13%), France (11%), China (11%), Italy (10%), Turkey (6%) and the USA (5%) are the top 6 vine-growing countries (Fig. 1a), representing 56% of the total area planted with vines. World wine production has been relatively stable over the past years (Fig. 1b). In 2021, it is estimated to be 260 million hectolitres (mhl), a decline of around 3 mhl (−1%) from 2020 (OIV, 2022).

By-products of the viticulture and winemaking processes include

vine shoots, grape pomace (including seeds, stalks, and skins), and wine lees. In addition to these waste products, wineries produce a significant volume of wastewater as well as an excess of grape must (Fig. 1c) (Beres et al., 2017; Contreras et al., 2022; Mäkelä et al., 2017; Zhang et al., 2017). Vine shoots (VS) from the agronomic practice of pruning account for up to 93% of winery leftovers (Sánchez et al., 2002). VS are composed of cellulose, hemicellulose, and lignin and can be considered as a platform synthesis of a plethora of biobased products such as oligosaccharides, proteins, lactic acid, bioactive compounds, bio-surfactants, xylitol, and biofuels such as ethanol and biogas (Cortés-Camargo et al., 2016; Dávila et al., 2016; Garita-Cambronero et al., 2021; Jesus et al., 2017, 2019; Pachón et al., 2020; Pérez-Rodríguez et al., 2016; Rajha et al., 2014; Rivas et al., 2007). In

* Corresponding author. CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal.

E-mail address: luciliad@deb.uminho.pt (L. Domingues).

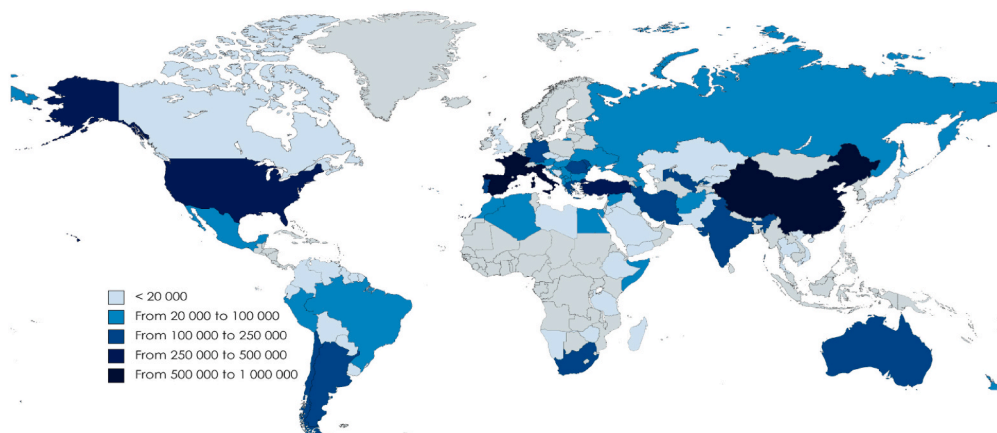
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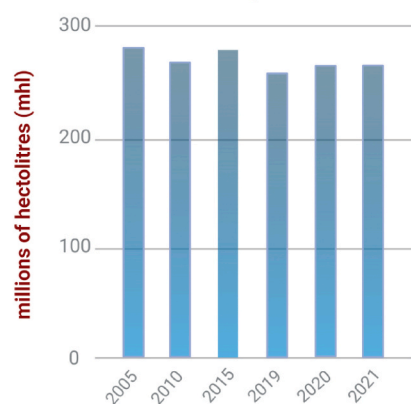
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a.

World vineyard distribution in 2021

b.

World wine production

c.



d.

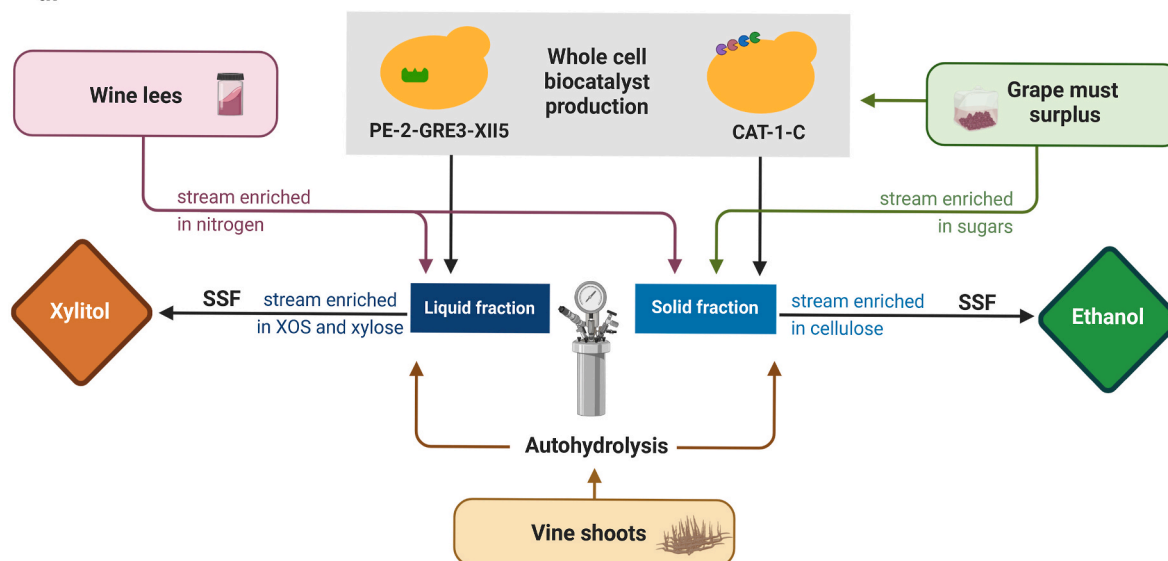


Fig. 1. (a) The world vineyard surface area in 2021, (b) global wine production in 2005, 2010, 2015, 2019, 2020 and 2021, (c) representation of wastes resulting from wine industry. Based on International Organisation of Vine and Wine (OIV) data 2005–2021. (d) A graphical representation of the integrated process developed for the valorization of waste from the wine industry. PE-2-GRE3-XII5 is the genome-engineered strain for the overexpression of GRE3 exhibiting increased production of xylitol. CAT-1-C was engineered to have cellulase enzymes displayed at the cell surface and was used to produce ethanol. This figure was created using Mapchart (<https://www.mapchart.net/>) and BioRender (<https://biorender.com/>).

this sense, using VS as a source of energy and value-added products rather than combusting or disposing on the ground to decompose is a more cost-effective and environmentally friendly option.

Wine lees (WL) are a common winery waste that forms at the bottom of wine containers after fermentation. It is composed of settled yeast cells and residual ethanol, representing 6% of wine the volume (Devesa-Rey et al., 2011; Pérez-Bibbins et al., 2015). WL have been used to recover ethanol by distillation, extract tartaric acid and phenolic compounds, and produce biogas and medium-chain carboxylates (Bustamante et al., 2008; Da Ros et al., 2014; Kucek et al., 2016; Rivas et al., 2006; Romero-Díez et al., 2018). Furthermore, WL have been evaluated for culture media supplementation in biotechnological processes due to their high protein and nitrogen content, as well as the presence of vitamins and essential amino acids (De Iseppi et al., 2020; Dimou et al., 2015; Hijosa-Valsero et al., 2021a).

The wine sector in Europe is strictly regulated with control measures to address the recurrent overproduction of wine in recent decades. One of the several measures adopted by the European community is the distillation of remaining wine for the production of cognac, vinegar, and other consumable foods and beverages (Corsinovi and Gaeta, 2019; Hirai, 1993; Meloni and Swinnen, 2013). The use of grape must surplus (GM) for the biosynthesis of polyols, mannitol and erythritol was recently proposed as a business opportunity for producers to get rid of excess wine production (Hijosa-Valsero et al., 2021a, 2021b). Given the high sugar content of GM, namely fructose and glucose, using this low-cost by-product as a substrate for cell growth could contribute to the circular economy and lower the overall cost of the process. The elimination of the various residues produced at different stages of grape and wine production is becoming not only an environmental concern but also an economic issue, which may reduce the sector's competitiveness (OIV: Collective Expertise, 2018).

Biorefining is considered one of the enabling technologies of the circular economy, aiming at the valorization of a wide variety of biomass, including agriculture residues, into a broad range of products and energy (Lindorfer et al., 2019). The establishment of biorefineries producing high-value chemicals in addition to low-value biofuels may reduce the use of non-renewable fuels and provide the required financial incentive to drive the development of biorefining technologies (Baptista et al., 2021; Bozell and Petersen, 2010).

With the remarkable progression of synthetic biology and metabolic engineering, the yeast *Saccharomyces cerevisiae* has become a reliable cell factory for sustainable production processes like second-generation ethanol and several bio-based chemicals (Baptista et al., 2021; Cunha et al., 2020b). It is crucial to select the appropriate yeast chassis to engineer, taking into account the target compound as well as the feedstock and process, in order to achieve high titers, yields, and productivity (Costa et al., 2017, 2021). Accordingly, Cunha et al. (2021) engineered a selected robust *S. cerevisiae* industrial strain to display cellulolytic enzymes on the cell surface. This strain was then used in consolidated bioprocessing to produce ethanol directly from lignocellulose. Additionally, recent advances in xylitol production by Simultaneous Saccharification and Fermentation (SSF) might be an attractive strategy for valorizing winery-derived wastes. An industrial *S. cerevisiae* strain, with an innate ability to accumulate xylitol (Romaní et al., 2015), was previously engineered for xylitol production by the overexpression of the endogenous aldose reductase (*GRE3*), which converts xylose into xylitol (Baptista et al., 2018), resulting in high xylitol titers from pretreated corn cob (Baptista et al., 2020) or hardwood xylan extracted with aqueous solutions of deep eutectic solvents (Romaní et al., 2020).

Taking all together and considering that a successful biorefinery deployment relies on the efficient conversion of a broad range of feedstocks into marketable products, here we propose an integrated biorefinery approach (Fig. 1d) that utilizes both liquid and solid winery waste streams to efficiently co-produce ethanol and xylitol. The product ethanol fulfills the global energy demand while xylitol increases the biorefinery profitability.

2. Material and methods

2.1. Strains, plasmids and construction of engineered strains

Escherichia coli DH5 α /NZY5 α (Nzytech, Portugal) was used for cloning work and plasmid maintenance. *E. coli* cells were grown at 37 °C, in Lysogeny Broth medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0) supplemented with 100 μ g/mL of ampicillin for transformant selection. All plasmids and primers used in this work are listed in Supplementary Material (Tables S1 and S2). Plasmid construction was performed by USER cloning, described by Jensen et al. (2014). The integrative plasmid (p2909_TEF-1_GRE3) was constructed by inserting the *GRE3* gene from pGRE3 (Baptista et al., 2018) into pCfB2909, an EasyClone-MarkerFree integrative vector without any selection marker (Jessop-Fabre et al., 2016). Sanger sequencing, to confirm correct cloning, was purchased from Eurofins Genomics. Yeast transformation was performed following the lithium acetate method (Gietz and Schiestl, 2007). The industrial *S. cerevisiae* PE-2 and CAT-1 strains, isolated from a first-generation bioethanol plant (Pereira et al., 2014), were used as chassis strains in this work. The PE-2 strain was transformed with a Cas9-expressing plasmid before further modifications. The transformants were selected in YPD media (10 g/L yeast extract, 20 g/L peptone, 20 g/L of glucose) supplemented with agar (20 g/L) and geneticin G418 (200 μ g/mL). Subsequently, the guide RNA (gRNA) helper vector targeting the XII-5 integration site (pCFB3050) was transformed along with the constructed integrative vector (p2909_TEF-1_GRE3) in the strain expressing the Cas9 protein, generating the strain PE-2-GRE3-XII5. Gene integration was confirmed by colony PCR.

CAT-1 was previously engineered to display *Aspergillus aculeatus* β -glucosidase I (EC 3.2.1.21; BGL1), *Trichoderma reesei* endoglucanase II (EC 3.2.1.4; EGII), *Talaromyces emersonii* cellobiohydrolase I (EC 3.2.1.91; CBH1) and *Chrysosporium lucknowense* cellobiohydrolase II (EC 3.2.1.91; CBH2) resulting in a robust strain (CAT-1-C) with hydrolytic activity for the direct ethanol production from cellulose (Cunha et al., 2021).

2.2. Raw materials

Vine shoots (*Vitis vinifera* L.), grape must (variety White Verdejo) and wine lees (variety Red) were kindly provided by the Center of Biofuels and Bioproducts, Agrarian Technological Institute of Castilla and León (ITACyL). Grape must (GM) and wine lees (WL) were kept at -20°C until use.

Vine shoots (VS) were analysed for extractives, carbohydrates and lignin following the NREL (National Renewable Energy Laboratory) procedures, as described in Romaní et al. (2016). GM and WL were previously analysed elsewhere following the procedures described by Hijosa-Valsero et al. (2021a).

2.3. Autohydrolysis pretreatment of vine shoots

Vine shoots (VS) were submitted to a hydrothermal treatment also known as autohydrolysis. The raw material was mixed with water at a liquid-solid ratio (LSR) of 4 and 6 (kg water per kg of oven-dry vine shoots) and heated to a maximal temperature (T_{max}) of 210–220 °C in a 2 L stainless steel reactor (Parr Instruments Company). The pretreatment severity (S_0) was calculated according to Baptista et al. (2020). After pretreatment, solid-liquid separation was performed by filtration and the solid fraction (pretreated VS) was recovered and washed for Solid Yield (SY) determination. The chemical composition of raw material and pretreated vine shoots was analysed according to NREL protocols (NREL/TP-510-42618-42622-4218). Acetyl groups and oligosaccharides in the liquid fraction were determined by acid post-hydrolysis of one aliquot of liquor (4% w/w H_2SO_4 at 121 °C for 20 min). Sugars, furans and acetic acid were quantified by HPLC (analytical conditions

described in section 2.7). The hemicellulosic hydrolysate was concentrated by a vacuum rotatory evaporator following the conditions reported by Domínguez et al. (2021).

2.4. Enzymatic saccharification of vine shoots hydrolysate

For enzymatic saccharification of the liquid fraction obtained from the hydrothermal treatment (vine shoots hydrolysate), 30 mL of hydrolysate were mixed with 75–600 μ L of cellulase cocktail (cellulase, enzyme blend [Cellic CTec2], Sigma-Aldrich) to achieve an Enzyme to Substrate Ratio (ESR) in the range of 88–350 U/g. The mixture was incubated at 30 °C, 200 rpm in an orbital shaker.

Glucose (GY) and xylose (XY) yields can be calculated via Eq. (1) and Eq. (2):

$$GY (\%) = \frac{G}{\frac{180}{162} f B} 100 \quad (1)$$

where G is the concentration of glucose (g/L), B is dry vine shoots biomass concentration (g/L), f is glucan fraction in dry biomass (g/g) and 180/162 (1.11) is the stoichiometric factor that converts glucan to equivalent glucose.

$$XY (\%) = \frac{X}{\frac{150}{132} (f \cdot B) + XOS + X} 100 \quad (2)$$

where X is the concentration of xylose (g/L), B is dry vine shoots biomass concentration (g/L), f is the sum of xylan fraction in dry biomass (g/g), XOS (xylooligosaccharides), and X (xylose) measured as xylose equivalent (g/L), 150/132 (1.13) is the stoichiometric factor that converts xylan to equivalent xylose.

2.4.1. Enzymatic activity determination

Cellulase and hemicellulase activities of Cellic CTec2 were 143 FPU/mL and 626 U/mL, respectively. For the measurement of xylanase activity in Cellic CTec2, the enzymatic cocktail was incubated with 10 g/L of xylan from beechwood (Sigma, $\geq 90\%$ purity) in 50 mM sodium citrate buffer (pH 5.0) for 10 min at 250 rpm orbital agitation at 30 °C. The amount of reducing sugar released from the substrate was measured by the DNS method (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme required to release 1 μ mol of reducing sugar per minute. Cellulase activity of the enzymatic cocktail was determined by the filter paper assay following the NREL protocol (NREL/TP-510-42,628) (Ghose, 1987).

2.5. Media and culture conditions

2.5.1. Pre-culture conditions

To evaluate the effect of grape must on yeast propagation, strains were grown in YPD and grape must-based media, at 300 rpm, 30 °C in 250 mL baffled shaker flasks with a working volume of 75 mL. Grape must-based media consisted of dilute solutions of grape must (GM), prepared by the addition of distilled water to a final concentration of 10%–50% of GM. GM was paper-filtered and sterilized in an autoclave (121 °C, 20 min) before dilution. For subsequent assays, yeast cells were grown on 50% white grape must (pH 5) for 24 h, and cell growth was scaled up to 1 L baffled shaker flasks with a working volume of 0.3 L. After propagation, cells were harvested by centrifugation (5 min, 3000 rpm, 4 °C), washed in 0.9% (w/v) NaCl solution and used to inoculate the culture medium with a concentration of 30–100 g of wet yeast/L. For the SSF experiment using cellulase-displaying CAT-1-C strain, the yeast was grown for 72 h to ensure an efficient cellulase expression (Cunha et al., 2021).

2.5.2. Batch cultivation for xylitol production

Production of xylitol by PE-2-GRE3-XII5 was performed in YP

medium (10 g/L yeast extract, 20 g/L peptone) with 15 g/L of glucose and 15 g/L of xylose and in vine shoots hydrolysate, previously saccharified by hemicellulase enzymes (section 2.4.), in 100 mL shake flasks (working volume 30 mL) at 30 °C and 200 rpm. For vine shoots hydrolysate experiments, the initial pH of the medium was set to 5.0 and CaCO_3 was added in stoichiometric proportion considering the concentration of potential acetic acid. Samples were periodically collected for sugar and xylitol determination by HPLC.

2.5.3. Simultaneous Saccharification and Fermentation of vine shoots hydrolysate for xylitol production – experimental design

The *S. cerevisiae* PE-2 strain genome-engineered to overexpress the GRE3 gene (section 2.1, PE-2-GRE3-XII5 strain) was used for xylitol production from xylose and xylooligosaccharides of hydrolysate in a Simultaneous Saccharification and Fermentation (SSF) process. The experiments were performed using concentrated vine shoots hydrolysate, in 100 mL shake flasks (working volume of 30 mL), at 200 rpm and 30 °C. The pH was adjusted as described above.

The full factorial design evaluated the independent variables: inoculum size and hemicellulase enzyme loading. The inoculum size (or x_1) ranged between 30 and 100 g of wet yeast/L and Enzyme to Substrate Ratio (ESR) or x_2 ranged between 159 and 477 U/g. The dependent variables, xylitol concentration and yield, were correlated with the independent variables by empirical models, following the polynomial expression:

$$y_j = b_{0j} + \sum_{i=1}^2 b_{ij}x_i + \sum_{i=1}^2 \dots \sum_{k \geq i}^2 b_{ikj}x_ix_k \quad (3)$$

where y_j ($j = 1$ to 2) is the dependent variable; x_i or x_k (i or $k: 1$ to 2, $k \geq i$) are the normalized, independent variables and $b_{0j} \dots b_{ikj}$ are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

2.5.4. Simultaneous Saccharification and Fermentation of pretreated vine shoots for ethanol production

The assays were performed in 100 mL shake flasks (working volume of 30 mL) with a glycerol lock to prevent oxygen entry and create oxygen-depleted conditions. Ethanol production was followed by the weight difference of Erlenmeyer flasks (associated with the release of carbon dioxide) as previously described (Costa et al., 2022; Cunha et al., 2019b, 2020a, 2021). The media consisted of 15% pretreated vine shoots (dry weight basis) supplemented with (i) 9% WL, (ii) 9% WL and 33% GM, and (iii) 42% of GM (wet weight basis). Lees and must were sterilized by autoclave (20 min, 121 °C) before addition. All media were adjusted to pH 5.0, as described above. Cellic CTec2 was added in a final concentration of 6 FPU/g of solids. The assays were performed in an orbital shaker at 150 rpm and 40 °C.

2.6. Fermentation parameters

Xylitol yield from xylose ($Y_{XyOL/Xy}$) was calculated by the ratio between produced xylitol and consumed xylose. Xylitol yield from xylan ($Y_{XyOL/XyAN}$) was calculated by the ratio between xylitol concentration (g/L) at the end of the SSF assays, and the potential xylose concentration (g/L) in the used media, multiplied by 100. The potential xylose was calculated by multiplying the xylan concentration by the stoichiometric factor that converts xylan to equivalent xylose (150/132) and by adding the XOS concentration in the liquor.

Ethanol yield ($Y_{EtOH/gluAN}$) was calculated by the ratio between the ethanol concentration (g/L) at the end of the SSF assays, and the potential glucose concentration (g/L) in the used media, multiplied by the theoretical stoichiometric yield (0.511 g of ethanol that is produced per gram of glucose), and the final ratio value was multiplied by 100. The

potential glucose was calculated by multiplying the glucan concentration by the stoichiometric factor that converts glucan to equivalent glucose (180/162). Xylitol (Q_{Xt}) and ethanol (Q_{Et}) productivity (g/L-h) at the time (t) was calculated as the ratio between the xylitol and ethanol concentration (g/L), at the time (t), divided by that time (t).

2.7. HPLC analysis

Samples from the different assays were analysed for quantification of glucose, xylose, xylitol, acetic acid, ethanol, HMF and furfural by HPLC using a Bio-Rad Aminex HPX-87H column, operating at 60 °C, with 0.005 M H₂SO₄ and at a flow rate of 0.6 mL/min. For fructose and xylose separation and quantification, when simultaneously present, the samples were analysed by HPLC using a Bio-Rad Aminex HPX-87 P column, at 85 °C, with H₂O at 0.5 mL/min.

3. Results and discussion

3.1. Biorefinery scheme and chemical composition of winery wastes

To establish a sugar platform based on the use of by-products generated during winemaking processing and viticulture practices, vine shoots (VS), the surplus of grape must (GM), and wine lees (WL) were the waste resources considered as renewable carbon-based feedstocks. Fig. 1d displays an overview of the design proposed in this study for the integral valorization of these winery wastes into bio-based products (xylitol, ethanol, and biocatalysts), using the wide range of sugars (xylose, fructose, and glucose) present in these feedstocks. GM, mainly composed of fructose and glucose (Table S3, Supplementary Material), was evaluated as an inexpensive carbon source for whole-cell biocatalysts production. These biocatalysts are required for subsequent conversion steps involving the native aldose reductase (Baptista et al., 2018) and the heterologous cellulases (Cunha et al., 2020a), to produce xylitol and ethanol, respectively. WL, on the other hand, are nitrogen-rich residues (Table S3, Supplementary Material) that may be employed as a low-cost alternative to peptone and yeast extract to minimize total production costs. Thus, GM and WL having accessible carbon and nitrogen sources could be applied to ethanol and xylitol production processes without further processing. Despite the interesting composition of VS (Table S3, Supplementary Material), its recalcitrant lignocellulosic structure requires a pretreatment to extract fermentable sugars. VS has a polysaccharide content of 46% (Table S3, Supplementary Material), with glucan and xylan being the main sources of glucose and xylose, respectively. This chemical composition is consistent with the literature (Dávila et al., 2016; Jesus et al., 2017).

Most research studies have focused on the valorization of a particular winery waste (Ahmad et al., 2020; Contreras et al., 2022; Jesus et al., 2022). Comparatively, limited research focuses on multi-waste valorization for the synthesis of more than one product (Filippi et al., 2022). Therefore, with the ultimate goal of enhancing the feasibility and sustainability of an integrated biorefinery while protecting the environment and promoting the circular economy, this study proposes a novel configuration process (Fig. 1d) to integrate multiple waste streams for the production of multiple products.

3.2. Grape must surplus as feedstock for whole-cell biocatalysts production

Industrial production of yeast cell biomass requires the efficient replication of cells to maximize yields and productivity, along with an optimum yeast product performance in subsequent industrial applications. Low-cost substrates that are easily accessible in large quantities are required to ensure good process economics. These substrates should have high concentrations of carbon compounds capable of being completely converted to cell biomass (Attfield, 2022). Sugar-rich grape must (GM) fits these requirements to become a preferred substrate for

the production of industrial yeast biomass. However, conditions of propagation have been shown to affect the cellular performance of *S. cerevisiae* (van Dijk et al., 2020). To evaluate the feasibility of utilizing GM as a carbon source, yeast cells were grown in aerated cultures with 10, 25, and 50% of grape must (Fig. 2). The data provided in Fig. 2a clearly shows that the growth kinetics was similar between the three tested conditions, and the biomass yield increased with increasing grape must concentration. Regardless of GM sugar consumption, in 42 h of cultivation, yeast cells consumed around 80% of the sugar present in the GM-based media (Fig. S1., Supplementary Material). Using 50% of GM, the maximal biomass concentration of 5.2 g/L was attained after 24 h of cultivation, indicating that high substrate concentrations did not negatively affect microbial growth. Despite the efficient utilization of GM to produce yeast biomass, the viability of these cells as biocatalysts in bioproduction processes must be verified.

The robust PE-2 strain, when genetically engineered with a xylose consumption pathway (XR/XHD from *Scheffersomyces stipitis*), was found to have a natural propensity to accumulate xylitol (Costa et al., 2017; Romání et al., 2015) and has been previously used as chassis to overexpress the *GRE3* gene in an episomal expression vector (Baptista et al., 2018). Chromosomal gene integration provides advantages over plasmid-based expression (such as higher strain stability and reduced population variability) and the use of antibiotic-based selection markers in industrial strains is unfavorable due to the possibility of drug resistance spread (Jessop-Fabre et al., 2016). Thus, in this work, a novel PE-2-*GRE3*-XII5 recombinant strain was constructed by the integration of the *GRE3* gene in the yeast genome. Single-copy chromosomal expression is often weaker than multicopy plasmid expression. Nevertheless, gene expression is highly dependent on several components of the genetic construction, including promoters, ribosome-binding sites, and enhancers or activators (Saleski et al., 2022). The single integration in a diploid strain, with a strong promoter (*TEF1*), resulted in an equivalent xylitol conversion phenotype when compared with the plasmid-based construct (Fig. S2., Supplementary Material).

Thus, the capacity of the genome-engineered PE-2-*GRE3*-XII5 strain, pre-grown in synthetic (YPD) and in GM-based media, was compared in batch cultivations using xylose-containing medium, supplemented with glucose. This carbon source supplementation is needed to create co-factors and maintain energy since *S. cerevisiae* does not natively metabolize xylose. Glucose was consumed in less than 2 h by the YPD and GM grown cells (data not shown). After 20 h and 48 h of cultivation, cells propagated in YPD produced 12.3 and 14.3 g/L of xylitol, and yeast cells grown in GM-based media produced 10.7 g/L in 20 h and 13.4 g/L, respectively (Fig. 2b). No clear difference in xylitol production was observed between cells grown in different substrate sources, indicating that non-supplemented GM is a suitable substrate to be used for cell propagation and could be implemented as a renewable raw material.

In yeast production plants, the utilization of by-products to produce biomass has already been adopted. Molasses, derived from beet or cane processing, is the substrate of choice for biomass production at a reduced cost. However, molasses need to be supplemented with essential elements for yeast growth, such as nitrogen, magnesium, phosphate, and vitamins. In addition, the employment of molasses in a variety of different industrial uses, such as bioethanol and lipids production (Cardona and Sánchez, 2007; Díaz-Fernández et al., 2019; Pandey et al., 2022) has also contributed to increasing the price of the commodity, which promotes the search for novel substrates for yeast biomass propagation (Attfield, 2022; Gómez-Pastor et al., 2011). Considering this, a possible outlet for surplus grape products is the use of GM as a replacement for molasses to yield yeast biomass in future integrated biorefineries that need to be able to expand and continuously diversify their feedstocks.

As 50% of GM allows the highest biomass yield while maintaining cell function, 50% was chosen as the optimum must concentration for yeast propagation experiments.

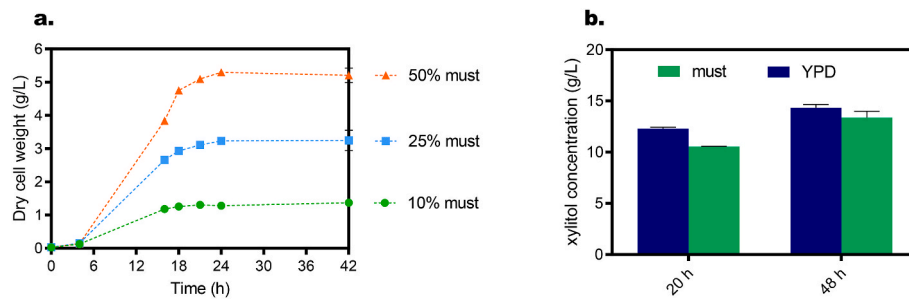


Fig. 2. Evaluation of grape must as a raw material for yeast growth. (a) Yeast biomass production using (●) 10%, (■) 25% and (▲) 50% of grape must. (b) Xylitol production at 24 h and 48 h by yeast previously grown in grape must-based media (green) and YPD media (blue). When the error bar is not shown, it is smaller than the corresponding data symbol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Vine shoots (VS) for ethanol and xylitol production: VS pre-treatment

Hydrothermal processing, also known as autohydrolysis, is commonly used for the solubilization of hemicellulose into oligosaccharides as the first step in a biorefinery (del Río et al., 2019), generating a residual biomass mostly constituted of cellulose and lignin (Romaní et al., 2010). The severity range of hydrothermal treatment was selected based on the literature (Jesus et al., 2017). The operational conditions and main results obtained (liquid and solid phases compositions) were listed in Table 1. According to the xylan content in the raw material, xylan was the highest solubilized polymer, with up to 78.5% of solubilization. The recovery of glucan after pretreatment ranged from 62.6 to 76.3 g glucan/100 g glucan in raw material. These glucan recovery values were lower than those of other hardwoods (such as Eucalyptus and Paulownia woods subjected to autohydrolysis pretreatment)

Table 1

Chemical composition of solid and liquid fractions resulting from the hydrothermal pre-treatment of vine shoots (VS).

T _{max} (°C) or S ₀ (–)	210 or 3.70	215 or 3.89	220 or 3.99	215 or 3.89	
LSR (g/g)	6	6	6	4	
Solid yield (g/100 g of VS)	60.2	58.5	60.2	59.8	
a) Solid fraction (g of component/100 g of pretreated VS)					
Glucan	33.3 ± 0.01	39.7 ± 0.05	40.6 ± 0.07	37.4 ± 0.18	
Xylan	8.13 ± 0.10	6.89 ± 0.06	4.89 ± 0.01	9.56 ± 0.12	
Acetyl groups	1.19 ± 0.02	0.91 ± 0.01	0.78 ± 0.00	1.89 ± 0.02	
Klason Lignin	45.0 ± 0.90	43.4 ± 1.06	47.3 ± 0.04	43.9 ± 1.21	
b) Liquid fraction (g/L)					
Glucose	0.81 ± 0.04	0.76 ± 0.05	1.0 ± 0.02	0.84	C.H. 2.21
Xylose	1.35 ± 0.01	2.21 ± 0.04	2.64 ± 0.01	1.60	3.55
Acetic acid	1.98 ± 0.08	2.76 ± 0.11	3.87 ± 0.18	0.64	1.02
Hydroxymethylfurfural (HMF)	0.34 ± 0.00	0.52 ± 0.01	0.81 ± 0.00	n.d.	0.19
Furfural (F)	0.39 ± 0.01	0.96 ± 0.00	1.85 ± 0.02	n.d.	0.17
Glucoligosaccharides (GOS)	16.82 ± 0.3	16.13 ± 0.5	13.51 ± 0.22	26.40	58.81
Xylooligosaccharides (XOS)	15.41 ± 0.3	15.56 ± 0.6	12.81 ± 0.1	25.07	57.15
Acetyl groups	4.15 ± 0.02	4.04 ± 0.01	3.59 ± 0.02	6.29	13.66

S₀: Severity.

LSR: liquid-solid ratio.

C.H.: concentrated hydrolysate, composition after evaporation.

n.d.: not detected.

(Domínguez et al., 2017; Romaní et al., 2010). This indicates that some glucan was solubilized as glucooligosaccharides, as verified by chemical analysis of the liquid fraction (Table 1). As a result, hydrothermal treatment conditions were favorable for obtaining a hemicellulosic hydrolysate enriched in oligosaccharides (26.3–32.2 g/L, calculated as the sum of glucooligosaccharide, xylooligosaccharide, and acetyl groups). The maximal concentration of potential xylose (17.7 g/L, calculated as the sum of xylose and xylooligosaccharides) was achieved at 215 °C (S₀ = 3.89). The temperature increase to 220 °C led to the reduction of oligosaccharides in the hydrolysate and an increase in sugar degradation compounds, such as furfural and hydroxymethylfurfural (HMF). Among the tested conditions, the hydrolysate obtained at 215 °C is more suitable for the production of xylitol. Furthermore, it is important to consider the glucose, in the form of glucooligosaccharides, that may be derived from hemicellulosic hydrolysate since the engineered strain requires a co-substrate for the xylose to xylitol conversion process (Baptista et al., 2018, 2020).

3.3.1. Ethanol production from pretreated VS

The cellulolytic *S. cerevisiae* CAT-1-C strain, displaying the *Aspergillus aculeatus* β-glucosidase 1 (BGL1), *Trichoderma reesei* endoglucanase II (EGII), *Talaromyces emersonii* cellobiohydrolase I (CBH1) and *Chrysosporium lucknowense* cellobiohydrolase II (CBH2) on the cell surface (Cunha et al., 2021), was evaluated for ethanol production in an SSF assay using pretreated VS as substrate. To achieve high ethanol yields, SSF experiments with the recombinant strain CAT-1-C requires supplementation with Cellic Ctec2 at low concentration of 6 FPU/g of solids (Cunha et al., 2021).

Previous research on ethanol production from hydrothermally treated vine shoots produced 19.1 g/L (from a solid obtained at S₀ of 4.60, using 16% of solids and 15 FPU/g) (Jesus et al., 2017) and 13.3 g/L (S₀ = 4.65, using 10% of solids and 20 FPU/g) (Dávila et al., 2019). Considering the hydrolytic capability of the recombinant strain, here a small dosage of commercial Cellic Ctec2 (6 FPU/g of solids) was added to the SSF experiments.

Hydrothermal treatment of hardwood biomass (such as eucalyptus, paulownia or vine shoots) is remarkable for its high solubilization selectivity for hemicelluloses. Nonetheless, the appropriate conditions for XOS production are insufficient for high cellulose-to-glucose conversion. To achieve high glucose concentrations and, subsequently, high ethanol yields from hardwood, a delignification process is usually applied to increase the glucan content and improve the enzymatic convertibility of glucan into glucose (Romaní et al. 2019; del Río et al., 2020). As an alternative to this costly and hazardous delignification process, GM-derived sugars may be used to boost the sugar concentration and, consequently, final ethanol concentration. This would follow the same line of thought as cheese whey incorporation in lignocellulosic-to-ethanol valorization processes (Cunha et al. 2018, 2021; Gomes et al., 2021), with the advantage of being composed of sugars that are readily consumed by the yeast. Thus, to examine the

effect of GM and WL incorporation in the SSF for ethanol fermentation, different mixture combinations of 15% of pretreated VS with grape must, wine lees, and grape must plus wine lees were evaluated (Fig. 3). The addition of GM, as well as GM in combination with WL, showed similar results. As expected, glucose and fructose from GM were consumed in less than 8 h in both experiments (Fig. 3a and b). The glucose accumulation after 24 h of SSF (Fig. 3b) indicates a loss of glucose uptake by the yeast cells, probably due to loss of fermentative capacity, limiting further ethanol production. A maximal concentration of 50.5 g/L of ethanol was obtained when GM has integrated alone with pretreated VS in the SSF process (corresponding to an ethanol yield of 0.32 g/g). The incorporation of WL together with GM also yielded more than 4% w/w, the critical threshold for the economic feasibility of the distillation process (Huang and Percival Zhang, 2011). However, the additional supplementation with WL does not significantly improve the fermentation performance. Compared to the SSF of VS supplemented with WL, which yielded 10 g/L of ethanol (0.16 g/g), the integration of GM led to a 3.7-fold increase in ethanol in SSF supplemented with GM and WL, and a 4.9-fold increase in SSF supplemented with GM.

3.3.2. Xylitol production from VS hydrolysate

- Enzymatic saccharification and fermentation of Vine Shoots hydrolysates

Hydrothermal treatment yielded a liquid fraction enriched in glucooligosaccharides (GOS) and xylooligosaccharides (XOS), which may be broken down into fermentable sugars by the action of enzymes. Enzymatic hydrolysis of these oligosaccharides offers multiple advantages over acid hydrolysis, including softer conditions, fewer inhibiting chemicals produced, and no neutralization before fermentation (Hu et al., 2016). Moreover, enzymatic approaches using commercial enzyme preparations have been used to deconstruct hemicellulose-derived oligomers to pure xylose and glucose (Baptista et al., 2020; Hu et al., 2016; Oliveira et al., 2018).

Saccharification experiments were performed on VS hydrolysate obtained at S_0 of 3.89. The release of glucose and xylose using a commercial enzymatic cocktail, Cellic Ctec2 (626 U/mL of hemicellulose activity) was monitored over 48 h. This cellulase mixture was selected due to its high hemicellulose activity, tolerance to inhibitory compounds (oligosaccharides and phenolic compounds) and higher thermostability (Hu et al., 2016). Although the vine shoot hydrolysate contained similar amounts of potential xylose (17.8 g/L) and glucose (16.9 g/L), the hydrolysis of xylooligomers was more effective in all tested conditions (Fig. 4). In addition to xylose and glucose monomers, the hydrolysis of acetyl groups attached to XOS releases acetic acid, an inhibitory product that negatively affects yeast growth (Arneborg et al., 1995; Cunha et al., 2019a; Phowchinda et al., 1995). Using a high ESR (350 U/g of

oligomers), the highest xylose yield was achieved after 6 h of enzymatic saccharification (90%, based on the potential xylose concentration) (Fig. 4a). It is possible to achieve more than 80% XOS hydrolysis after 48 h with lower ESR (175 U/g and 88 U/g), but industrial processes benefit from a rapid and efficient conversion. The use of high enzyme loadings clearly affected the glucose production, ESR of 175 and 350 U/g led to a 1.5 and 3.9-fold increase in glucose concentration after 24 h of saccharification, respectively.

As above mentioned, the genome-engineered PE-2-GRE3-XII5 yeast strain is a non-xylose-consuming organism and therefore needs to be supplied with a carbon source. Considering this, the VS hydrolysate attained at S_0 of 3.89 and saccharified using 350 U/g of enzyme is an appropriate renewable substrate for xylitol production since it can provide xylose for bioconversion as well as glucose for cell growth. Fig. 5 shows the evaluation of VS hydrolysate for xylitol production by the novel genome-engineered PE-2-GRE3-XII5 strain. Despite the initial concentration of 5.6 g/L acetic acid in the medium (Fig. 5a), the strain could convert 75% of xylose to xylitol with a corresponding yield of 0.98 g/g. Acetic acid is the main inhibitor found in VS hydrolysate. Previous research has shown that acetic acid may completely inhibit cell growth of xylose-consuming yeasts, including *Candida guilliermondii*, *C. tropicalis* and *C. boidinii* (Pereira et al., 2011; Romero-García et al., 2022; Wang et al., 2013). The yeast *S. cerevisiae*, on the other hand, has an innate metabolic ability to overcome acetic acid-mediated inhibition of lignocellulosic hydrolysate fermentation (Mira et al., 2010). A recent study developed a biological treatment with *S. cerevisiae* for detoxifying acetic acid produced during the processing of lignocellulosic olive stone in order to increase the productivity of *C. boidinii*, which was employed to convert lignocellulosic-derived xylose to xylitol (Romero-García et al., 2022). In addition, robust *S. cerevisiae* isolates, such as the chassis strain used in this work, have shown increased tolerance to inhibitors (Pereira et al., 2014; Costa et al., 2017). Still, robust yeast strains were shown to differentially express tolerance genes (for instance, *PRS3*, *RPB4* and *ZWF1*) in response to different lignocellulosic inhibitor loads (Cunha et al., 2015). In spite of the robustness of the yeast strain used as host strain, the acetic acid produced during the enzymatic hydrolysis of corn cob hydrolysate, if higher than 4.3 g/L, reduced xylitol productivity (Baptista et al., 2020). Taking into account our previous findings, as well as the fact that yeast cells can adapt to weak acids in a glucose-containing medium with a pH of 4.5 and grow after a lag phase (Giannattasio et al., 2013), the addition of calcium-carbonate (CaCO_3) in the hydrolysate to buffer the medium was also investigated.

As observed in Fig. 5b, the recombinant strain when cultured in media supplemented with CaCO_3 was able to convert almost all the sugar in 24 h, producing 12 g/L of xylitol at a yield of 0.96 g/g. This methodology led to an increase in xylitol productivity (maximal 0.56 g/(Lh)), a 1.5-fold improvement over cultures without CaCO_3 supplementation.

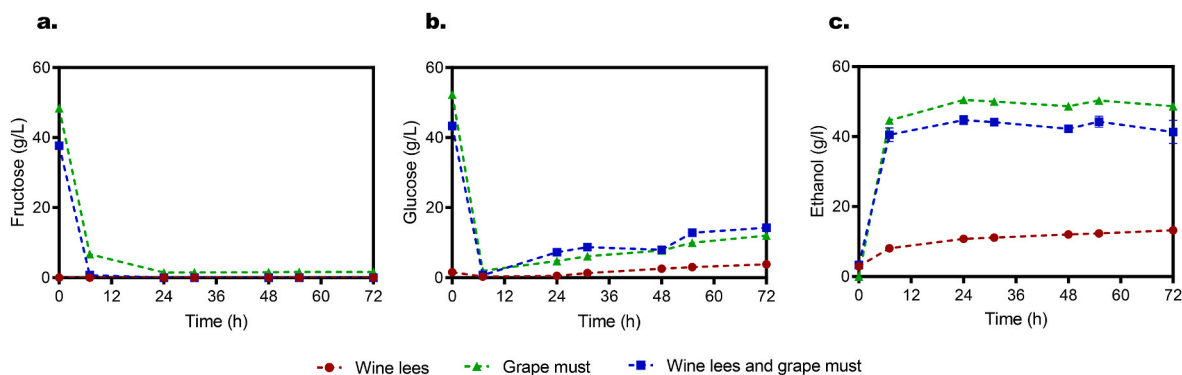


Fig. 3. Simultaneous saccharification and fermentation of vine shoots solid residue (15%) supplemented with wine lees (WL) (●), wine lees (WL) and grape must (GM) (■), and grape must (GM) (▲) by the CAT-1-C strain. Profiles of (a) fructose, (b) glucose and (c) ethanol concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

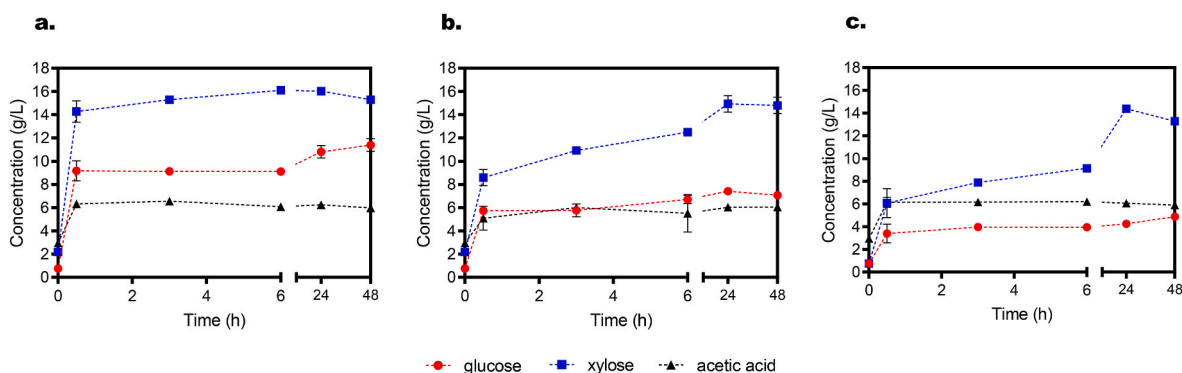


Fig. 4. Enzymatic saccharification of vine shoots hydrolysate from autohydrolysis at 215 °C using the enzyme to substrate ratio (ESR) fixed at (a) 350 U/g, (b) 175 U/g and (c) 88 U/g. Profiles of glucose (●), xylose (■) and acetic acid (▲) concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

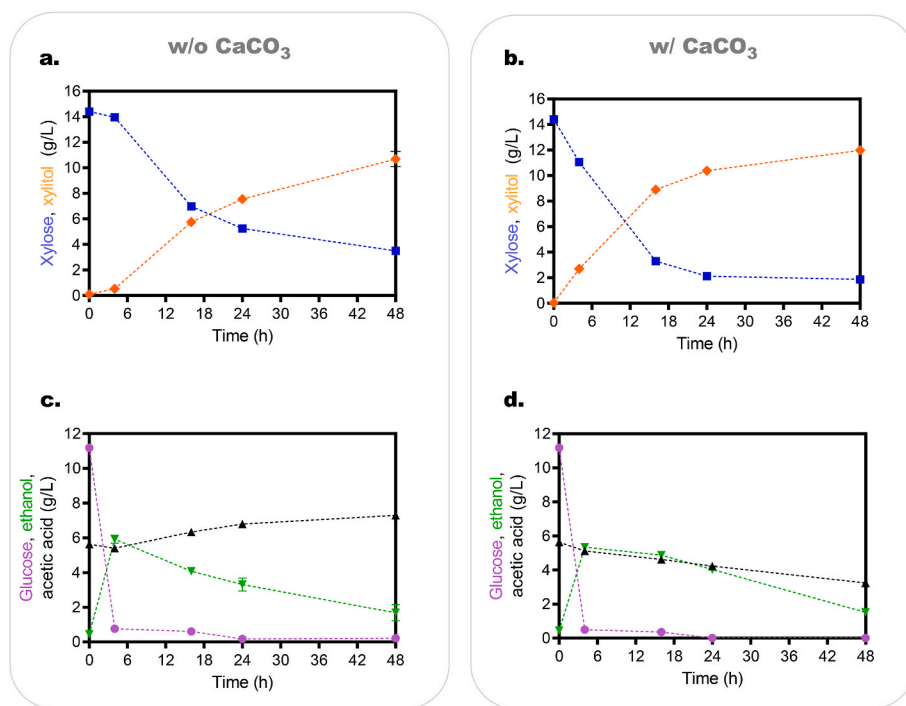


Fig. 5. Time course of xylose (■) and xylitol (♦) (a,b), and glucose (●), ethanol (▼) and acetic acid (▲) (c,d) concentrations in the enzymatically hydrolysed vine shoots hydrolysate with (a,c) and without (b,d) CaCO_3 for the novel genome-engineered PE-2-GRE3-XII5 strain. Each data point represents the average $\pm$ standard deviation of biological duplicates. When the error bar is not shown, it is smaller than the corresponding data symbol.

In contrast to the accumulation of acetic acid observed in the medium without CaCO_3 (Fig. 5c), it was possible to observe a slight consumption of acetate during cultivation with CaCO_3 (Fig. 5d). In fact, it has been suggested that the toxic effects of acetic acid accumulated in the culture medium have a role in the process of chronological ageing in yeast (Burtner et al., 2009), and buffering the medium can extend the chronological life span (Weinberger et al., 2010). Related results were obtained in lactic acid fermentation of brewer's spent grain hydrolysate, where CaCO_3 addition increased volumetric productivity using *Lactobacillus rhamnosus* (Pejin et al., 2015).

- Experimental design for xylitol production from vine shoots hydrolysate by SSF

Considering the results obtained from hydrothermal treatment, an additional experiment at 215 °C reducing the LSR to 4 g/g was carried out to increase the potential xylose (xylooligosaccharides and xylose) for

further conversion into xylitol (Table 1). The hemicellulosic hydrolysate was enriched up to 26.7 g/L of potential xylose and 27.3 g/L of potential glucose, resulting in a 1.6-fold increase in sugar concentration when compared to the hydrolysate generated at LSR of 6 g/g. Acetic acid and acetyl groups were also increased up to 6.3 g/L.

To increase the sugar content and remove volatile components, vacuum evaporation was applied to VS hydrolysate, as previously proposed by Domínguez et al. (2021) for *Paulownia* wood-derived hydrolysate. Following processing, the concentrated hydrolysate contained 58.8 g/L of GOS, 57.2 g/L of XOS, and 13.7 g/L of acetyl groups (Table 1).

To assess the capacity of the novel genome-engineered PE-2-GRE3-XII5 strain to produce xylitol from a non-supplemented and concentrated VS hydrolysate in an SSF process, a complete factorial design was proposed to study the variables: i) inoculum size and ii) enzyme loading. The concentrated VS hydrolysate (66%), corresponding to 40 g/L of potential xylose, was used as fermentation media. The experimental

matrix and main results obtained (xylitol concentration and xylitol yield) are listed in Table 2. During the enzymatic saccharification of VS hydrolysate, the hydrolysis of acetyl groups linked to XOS released quantities of acetic acid ranging from 24.8 to 30.1 g/L (Fig. S2, Supplementary Material). High enzyme loading (runs 10 and 11) and a large inoculum size produced the highest xylitol concentrations (24.1 and 24.3 g/L). This supports the already reported correlation between high initial cell biomass and improved tolerance to the stress induced by hydrolysate-derived inhibitors (Palmqvist and Hahn-Hägerdal, 2000; Wallace-Salinas and Gorwa-Grauslund, 2013).

Since experimental design is a useful tool for determining the influence of independent variables on dependent variables as well as optimizing conditions, a correlation analysis was performed on the experimental variables using equation (3). The regression coefficients, model significance, and statistical significance for xylitol concentration, productivity, and yield are included in Table 2. As seen, linear terms for variable x_1 (inoculum size) and x_2 (hemicellulose loading-ESR) and the combination of inoculum size and hemicellulase loading were significant at 90, 99, and 95% of confidence levels. Both independent variables studied had a positive effect on xylitol production, yield, and productivity.

Despite the positive results, the xylitol yields and productivities achieved in these experiments (Table 2) are far from the theoretical, probably due to the loss of fermentative capacity by the recombinant yeast.

To test whether nutritional supplementation could support yeast physiology maintenance, the concentrated VS hydrolysate was supplemented with commercial peptone (20 g/L) and yeast extract (10 g/L). Additionally, the addition of WL as a low-cost nitrogen source was also evaluated (Fig. 6). As can be observed, the addition of commercial nutrients (YP supplementation) permitted a faster and nearly complete conversion of xylose to xylitol, producing 37 g/L of xylitol (0.93 g/g of yield) (Fig. 6a). In contrast, WL supplementation had no positive influence on yeast performance, resulting in a similar production to those obtained without nutritional supplementation (run 11, Table 2). It should be noted that the release of acetic acid during the SSF supplemented with WL reached 20.5 g/L (Fig. 6d). Since the maximal acetic acid concentration that could be formed during VS saccharification is

14.7 g/L, the extra acetic acid appears as a by-product of yeast metabolism. The slower rate of conversion and lower xylitol production may have resulted from the release of this carboxylic acid, which was previously related to longer lag phases (Baptista et al., 2020). According to another study, the presence of components such as yeast extract provides some protection against the adverse stress effects of acetic and lactic acids (Narendranath et al., 2001). However, no systematic investigation has been conducted to determine if and which complex-medium components may protect yeast from these inhibitory effects.

Apart from being often used for the fermentation of cellulosic ethanol, SSF may be efficiently applied for xylitol production. Other studies that should not be compared to this study have reported the application of this process configuration for the valorization of corn cob (Baptista et al., 2018, 2020; He et al., 2021; Kogje and Ghosalkar, 2017) and corn stover (Yang et al., 2020).

3.3.3. Integrated biorefinery from wine waste

Winery leftovers account for 20% of total wine production (Arvanitoyannis et al., 2006; Zacharof, 2017). The high organic content of these wastes not only implies ecological concerns if discharged without further treatment but also provides a reservoir of carbon sources for chemicals and energy production. In comparison to the first-generation bioethanol industry, their integration into a second-generation biorefinery model would reduce the environmental impact of the wine industry through cost-effective waste valorization (Ioannidou et al., 2022; Moonsamy et al., 2022). Furthermore, multi-product biorefineries are being promoted (Baptista et al., 2021) rather than traditional biorefineries that focus on the production of a single product (Baptista et al., 2021; del Río et al., 2021).

Therefore, the selection of the most appropriate high-value products to complement the production of low-value biofuel in a biorefinery is crucial for achieving high profitability. The sugar-alcohol xylitol is included in the US Department of Energy list of high-value compounds that can be produced from biorefinery carbohydrates (Werpy and Petersen, 2004; Baptista et al., 2021) with a current market price of 7.95 €/kg (Vollmer et al., 2022). A recent techno-economic analysis of a lignocellulosic biorefinery for the production of xylitol coupled with

Table 2

Operational conditions of Simultaneous Saccharification of non-supplemented and concentrated Vine Shoots hydrolysate, and results obtained for dependent variables y_1 to y_3 . Regression coefficients, values and significance (based on a t -test).

Run	x_1	x_2	Inoculum (g of wet yeast/L)	Enzyme loading (U/g substrate)	Final xylitol concentration (g/L) or y_1	Productivity (g/(L·h)) or y_2	Xylitol yield (g/g) or y_3
1	-1	-1	30	159	18.2	0.13	0.300
2	0	-1	65	159	16.9	0.12	0.279
3	1	-1	100	159	15.6	0.11	0.257
4	-1	0	30	318	18.5	0.13	0.305
5	0	0	65	318	21.0	0.15	0.347
6	0	0	65	318	21.0	0.15	0.346
7	0	0	65	318	21.9	0.15	0.361
8	1	0	100	318	22.5	0.16	0.370
9	-1	1	30	477	19.7	0.14	0.324
10	0	1	65	477	24.1	0.17	0.396
11	1	1	100	477	24.3	0.17	0.400
coefficient				Xylitol concentration at 144 h		Xylitol productivity	
b_{0j}				21.363		0.1505	
b_{1j}				0.995 ^c		0.0067 ^c	
b_{2j}				2.868 ^a		0.02 ^b	
b_{12j}				1.8025 ^b		0.0125 ^a	
b_{11j}				-0.9428		-0.0063	
b_{22j}				-0.9428		-0.0063	
R^2				0.937		0.925	
F_{exp}				14.97		12.42	
Significance level (%)				>99		>9	

^a Coefficients significant at the 99% confidence level.

^b Coefficients significant at the 95% confidence level.

^c Coefficients significant at the 90% confidence level.

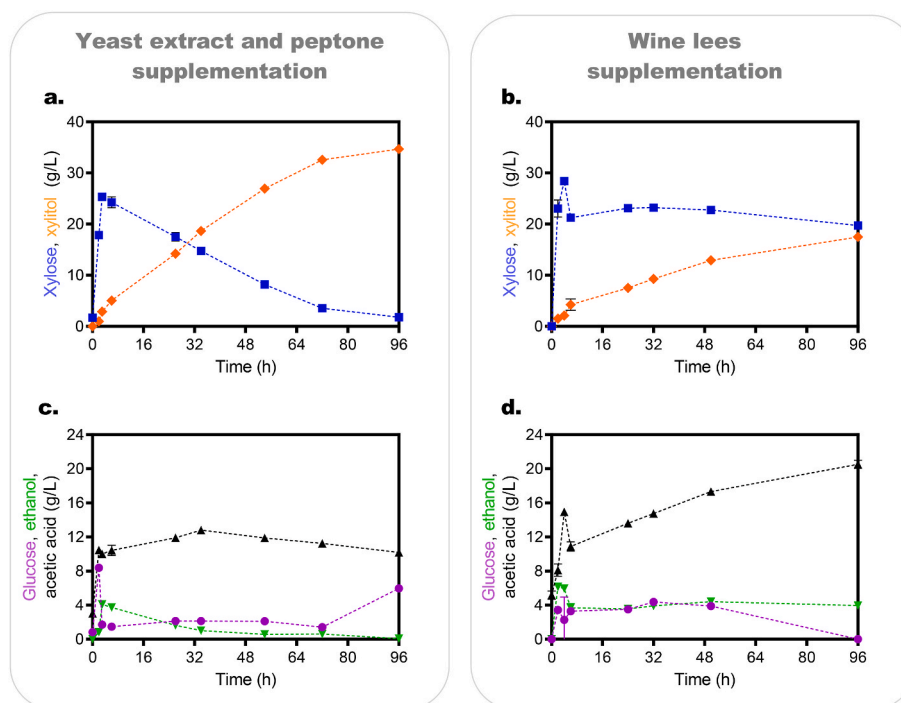


Fig. 6. Simultaneous saccharification and fermentation of concentrated vine shoots hydrolysate under optimal conditions (100 g of wet yeast/L of inoculum and 170 U of Cellic Ctec2) supplemented with (a) yeast with commercial yeast extract and peptone and (b) wine lees. Profiles of xylose (■) and xylitol (◆), glucose (●), acetic acid (▲) and ethanol (▲) concentrations. When the error bar is not shown, it is smaller than the corresponding data symbol.

second-generation ethanol revealed that integrating the biorefinery is more cost-effective than producing ethanol alone in terms of overall investment cost (Giuliano et al., 2018). Overall, the biorefining perspective proposed in this study allowed to obtain 37 g/L of xylitol and 50 g/L of ethanol in two separated streams through a valorization of multiple types of waste from the wine industry (Fig. 1). These results can be positively compared to those obtained from using winemaking wastes to produce one single product. In fact, the ethanol concentrations attained with this approach are higher than those previously reported for the fermentation of autoclaved grape must (10 g/L) (Williams et al., 2019) and hydrothermally treated and delignified vine shoots (20 g/L) (Senila et al., 2020). Furthermore, the xylitol titer is similar to the one previously reported from the sequential fermentation of grape shoots by *Lactobacillus acidophilus* and *Debaryomyces hansenii*, which yielded 31.3 g/L of xylitol but with a previous detoxification step (Portilla et al., 2008).

Annually, the required trimming of grape vines creates around 5 tonnes per hectare of grape cultivation (Dávila et al., 2017). Taking into account the results hereby obtained, besides ethanol approximately 67 kg of xylitol could be produced from 1 ton of vine shoots, signifying a worthy co-valorization route.

Other strategies using olive pruning (Mateo et al., 2015), olive stones (Saleh et al., 2014), and corn cob (Du et al., 2020) for co-producing xylitol and bioethanol have been recently developed, but winery wastes have not been evaluated before. Considering the environmentally friendly methodologies employed, which did not include harmful chemicals addition, as well as the superior concentrations achieved, the results obtained here can be compared favorably to those that have already been published in the literature.

4. Conclusions

This work provides an integrated novel biorefinery concept which aims at valorizing winery wastes. The utilization of grape must, produced in excess, showed to be feasible for yeast propagation and may lower overall process costs. To maximize the conversion of all

polysaccharides in vine shoots lignocellulosic biomass, hemicellulosic and cellulosic fractions resulting from vine shoots hydrothermal processing were converted into xylitol and ethanol, respectively. In the hemicellulosic-to-xylitol conversion process, a robust industrial isolate was genome-engineered for enhanced xylitol production and the experimental design demonstrated a significant effect of enzyme loading and inoculum size on the production process. Moreover, nutrient supplementation was shown to further overcome acetic acid inhibition. In the cellulosic-to-ethanol conversion process, the combination of vine shoots with surplus grape must enabled a sustainable production of ethanol, with an economically feasible distillation process. Furthermore, by applying a robust yeast chassis with cell surface display of cellulases, the enzyme added to the process was reduced. Still, the xylitol and ethanol titers attained in two different streams, 37 and 50 g/L, respectively, compare quite favorably with single-product wine wastes valorization processes.

Collectively, this strategy establishes the solid basis for the implementation of an integrated biorefinery by expanding the scope of winery multi-waste effective utilization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2022.116623>.

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