Vine shoots pre-treatment strategies for improved hydrogen production and metabolites redistribution in *Clostridium butyricum*

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Abstract

Physical pre-treatments by steam explosion (SE), chemical by organosolv (OS) and biological by laccase (LAC) were carried out in vine shoots to disrupt the cell fiber and increase the biomass hydrolysis and fermentation into hydrogen (H_2). After SE, there was a slight decrease in cellulose and hemicellulose contents in biomass fibers, while a decrease in lignin content occurred after OS pre-treatment. There were no quantifiable changes after laccase pre-treatment, however the enzyme-substrate oxidative reactions were favorable for hydrolysis and fermentation since an increase in soluble sugars and H_2 production was observed with LAC vine shoots as substrate. 300.1 mL H_2/L were obtained from raw material vine shoots, while 649.4, 399.8 and 749.7 mL H_2/L were obtained from biomass pre-treated by SE, OS and LAC, respectively. Furthermore, the hydrolysis of pre-treated biomass by addition of cellulase was evaluated to improve H_2 production. Higher amounts of H_2 were obtained from hydrolyzed biomass in relation to non-hydrolyzed ones (154.2, 602.0 and 167.1% more with SE, OS and LAC hydrolyzed, respectively). In all cases, the mixed acid pathway was carried out by *Clostridium butyricum*, since acetic and butyric acids were produced.

Keywords: Lignocellulosic biomass, steam explosion, organosolv, laccase, butyric acid, hydrogen

1. Introduction

Winemaking, one of the main agro-industrial activities in the world, had an estimated wine production of 260 million hectoliters in 2022, of which 157 million hl were produced in the European Union (EU) (OIV, 2022). Along with this intense production, the wine industry generates a large number of waste and by-products that are traditionally destined for composting and animal feed. (Bignami et al., 2022; Rani et al., 2020).

Among viticulture waste, vine shoots are the main waste generated during the vine pruning agronomic practice, with up to 2 tons of production per hectare annually (Sánchez-Gómez et al., 2017b). Vine shoots consist of cellulose (28-40%), hemicellulose (17-28%) and lignin (25-32%) in addition to extractives (~3%). This residue has low economic value and very limited use, and it is generally destined for burning and soil disposal (Contreras et al., 2022; Senila et al., 2020).

Seeking a more noble use, vine shoots can be used to produce polyphenolic compounds that are found as non-structural components or as part of lignin (Gullón et al., 2017). These vine shoot extracts have some potential purpose, such as in cosmetics, nutraceuticals or pharmaceuticals which are linked to their antioxidant effects, or in organic agriculture due to their anti-food and allelopathic activities (Sánchez-Gómez et al., 2017a). Along with polyphenols, Rajha et al. (2014) reported protein extraction by physical treatments of vine shoots.

Considering the fermentable portion of the biomass, hemicellulose (Dávila et al., 2016) and glucose (Dávila et al., 2017) can be recovered. El Achaby et al. (2018) reported the production of cellulose nanocrystals from vine shoots using chemical treatments followed by acid hydrolysis process. Senila et al. (2020) used vine shoots as substrate for second-generation bioethanol from simultaneous saccharification and fermentation. Therefore, this requires the optimization of autohydrolysis pre-treatment and chlorite delignification. The maximum bioethanol production (6%) was obtained by autohydrolysis (165 °C), chlorite delignification and SSF process at 37 °C, 10% solid loading (w/v), 72 h. For methane production from vine shoots, Pérez-Rodríguez et al. (2016) evaluated ultrasound pre-treatments and enzymatic hydrolysis of the biomass. The authors observed that ultrasounds were negative and the enzymatic hydrolysis was beneficial, increasing the methane production by 59.8%.

It was observed that, due to the complex structure of the vine shoots interconnected by strong covalent and noncovalent bonds and forces, a biomass pre-treatment step is required for its conversion into bioactive compounds, cellulose nanocrystals, bioethanol or methane. There is still no information about the best vine shoots pre-treatment for biological hydrogen (H_2) production.

The H_2 production as an alternative fuel is attracting interest in the context of global climate change. The direct combustion of H_2 does not emit carbon dioxide (CO_2), reducing dependence on fossil fuels and mitigating environmental impacts (Lepage et al., 2021). H_2 production methods can be divided according to the feedstocks used: fossil fuels (natural gas steam reforming, partial oxidation of heavy hydrocarbons, coal gasification), water (water electrolysis, photo-electrochemical and thermochemical processes), and biomass (biological processes, biomass gasification, ethanol steam reforming, and liquid-phase reforming).

H_2 production from fossil fuels can take advantage of the existing infrastructure for extracting, transporting, and storing these fuels. It can be carried out on a large scale, which can result in more efficient production in terms of quantity produced (Nahar et al., 2017). However, there is CO_2 emission, so environmental aspects need to be considered, and in this regard, H_2 production from renewable sources, such as water and biomass, is more attractive.

Water electrolysis and photo-electrochemical processes use renewable energy, resulting in low greenhouse gas emissions, but require significant amounts of electrical energy to break down the water molecules to obtain hydrogen. Therefore, the energy efficiency of these methods is a particular challenge, since some of the energy provided is dissipated in the form of heat during the process (Rasul et al., 2022).

H_2 production from biomass uses organic waste, reducing waste and contributing to waste management. In addition, the concomitant production of metabolites, such as organic acids and alcohols, can have industrial applications and be exploited as high value-added products (Rabelo 2018). There are challenges in efficiency, substrate selection, the need to optimize fermentation conditions, and economic feasibility (Camargo et al., 2023). Biological production is considered a promising way to produce H_2 providing a viable means for sustainable energy supply with low pollution and high efficiency (Ramprakash et al., 2022). Despite the challenges, hydrogen as an alternative fuel can play an important role in the transition to a low-carbon economy, requiring investment in research, development and infrastructure.

Pre-treatment by physical, chemical or biological means have been studied for the use of biomass in fermentation processes. To choose the pre-treatment, the production of a reactive cellulosic fiber for enzymatic attack should be considered. It prevents the destruction of hemicelluloses and cellulose, and the formation of possible biological inhibitors (Camargo et al., 2023). With so many requirements, one must carefully study the inherent factors of the biomass and also the cost-effective requirements.

In physical pre-treatments, chemical reagents or microorganisms are not needed to promote the breakdown of the lignocellulosic structure. Among them, steam explosion has been applied in sugarcane bagasse (Ratti et al., 2015), olive leaves (Romero-García et al., 2016), olive tree pruning (Oliva et al., 2020) to increase the release of soluble sugars, the production of hydrogen and methane, respectively. During steam explosion pre-treatment, the biomass is subjected to high temperatures and pressure for a short period of time inside a reactor. The pressure is then rapidly reduced, leading to disruption of the cellular fiber, facilitating the subsequent biological hydrolysis and soluble sugars fermentation (Yu et al., 2022). Romero-García et al. (2016) evaluated steam-explosion treatment as a procedure to recover sugars and natural antioxidants from olive leaves. Operating conditions were optimized and, with whole leaves, at 180 °C, 8.3 min, 70% of the initial sugars in the olive leaves were recovered with low formation of inhibitory compounds.

The chemical pre-treatment occurs through the reagents added to the biomass, whether acid, alkali or organic solvents, in order to change the physico-chemical characteristics of the biomass. Pre-treatments with addition of organic solvents, also known as organosolv, preferably use low-cost alcohols or mixed with water to remove lignin and hydrolyze hemicellulose. The formation of fermentation inhibitors is very low, and solvents must be removed before fermentation (Behera et al., 2014). The liquid fraction can be used to recover lignin with high purity and low molecular weight, phenolic compounds (Padilla-Rascón et al., 2022) or sugars for anaerobic digestion (Ostovareh et al., 2015). The solid fraction, composed of a cellular fiber that is less recalcitrant due to the partial breakage of lignin-hemicellulose bonds, can be used as a substrate in fermentation processes. Gómez-Cruz et al. (2021) reported the efficiency of organosolv pre-treatment (50% ethanol, 1% sulfuric acid, 130 °C, 60 min) for exhausted olive pomace delignification. Organosolv has already been used in pre-treatment of several other types of biomass such as sugarcane bagasse (Romero et al., 2010), wheat straw (Huijgen et al., 2012), bamboo (Li et al., 2012), palm oil tree residues (Alriols et al., 2009) and olive stones (Padilla-Rascón et al., 2022).

Biological pre-treatment uses a metabolite from a microorganism, and as no chemicals are used in this process, there is no need to recycle the chemical products, and it does not release toxic compounds to the environment. It is an environmentally friendly process, with few limitations in using this strategy for the pilot scale process (Camargo et al., 2023), and has gained much attention to generate biofuels/biochemicals (Rajak and Banerjee, 2018). Laccases are enzymes from a microorganism that act on substrates rich in aromatic compounds such as lignin and are widely used in lignocellulosic biomass pre-treatment (Avanthi and Banerjee, 2016). Furthermore, the laccase reaction in the lignocellulosic structure can also favor the biomass fermentation. Rajak et al. (2018) obtained higher ethanol production from kans grass subjected to the enzymatic partial hydrolysis/saccharification step followed by fermentation (63.2 g/L) than from kans grass subjected to simultaneous saccharification and fermentation (57.9 g/L). The authors related the increase in ethanol produced to the structural distortion of biomass due to enzymatic action. For sugarcane bagasse (SCB) fermentation, Rabelo et al. (2022) reported an 84% increase in the fermentative H_2 production (and 184% in the amount of H_2) after enzymatic pre-treatment with laccase (0.4 U laccase/mL, 24.0 g SCB/L and 120 min reaction at 37 ºC) when compared with sugarcane bagasse without pre-treatment.

Although a study with the use of vine shoots for biological H_2 production has not yet been reported, the importance of vine shoots pre-treatment is observed when it is used in fermentation processes for ethanol production (Dávila et al., 2019; Senila et al., 2020) or digestion in methane (Pérez-Rodríguez et al., 2016). Therefore, this work evaluates, for the first time, the possibility of using vine shoots as a substrate for the fermentative H_2 production, evaluating one type of each physical, chemical and biological pre-treatments in order to increase biofuel production. It also brings a complete characterization of the cellular fiber before and after each pre-treatment that will serve as a basis for future studies with the scope of reusing shoots in biological processes. This work is in line with the search for new possibilities to reuse vine shoots and the sustainable production of clean energy.

2. Material and methods

2.1. Biomass raw material

The vine shoots used as substrate were collected in Penedés region, Spain. The biomass was ground in a mill Retsch® model SM 100, (Haan, Germany), with a 1 cm sieve separator and rotational speed of 1500 rpm, into particles ≤ 1 cm. The biomass was air-dried to constant humidity (10.33 ± 0.12%), stored at room temperature and used in all pre-treatments tests.

2.2. Biomass pre-treatments

Due to the lack of studies with suitable pre-treatment conditions for vine shoots to be used as substrate for the fermentative H_2 production, the pre-treatment conditions for vine shoots were based on maximizing pre-treatment efficiency and minimum production of inhibitors fermentation processes, both requirements obtained with other types of biomass.

The physical pre-treatment of vine shoots was conducted in a custom-built steam explosion system (SE). The SE pilot unit (ATI® Sistemas, S. L., La Coruña, Spain) was equipped with a 4.5 L reaction tank designed to achieve high operating pressures (up to 40 bar). The reactor, previously preheated, was charged with 400 g of raw material (the one described in Section 2.1) and immediately heated again with saturated steam. The reactor was closed and, when it reached a temperature of 170 °C, for 5 min, it was suddenly depressurized. The selection of physical pre-treatment conditions with low severity aimed at the minimum production of inhibitors for subsequent fermentation (Romero-García et al., 2016). All pre-treated biomass (wet solid) was taken for characterization and fermentation.

The organosolv chemical pre-treatment (OS) was carried out at 15% (w/v) biomass and 50% (v/v) aqueous ethanol solution in a laboratory scale 1 L stirred tank reactor (Parr® Instrument Company, Moline, IL, USA). Biomass and ethanol were added to the reactor, which was closed and kept at 200 °C for 60 min under stirring. The OS process conditions (200 °C, 60 min, no acid) were selected based on previous studies with higher levels of delignification of wheat straw (Huijgen et al., 2012), olive stone (Padilla-Rascón et al., 2022) and olive tree biomass (Díaz et al., 2011). The pre-treated biomass was separated from the liquor by vacuum filtration, washed, characterized and used in the fermentation assays. The liquor was discarded due to the harmful alcohol content for bacterial growth.

The biological treatment was carried out with laccase enzymes (LAC) from *Trametes versicolor* (Sigma-Aldrich® 38429) with activity 0.7 U/mg. Laccase was diluted in 25 mL of citric-citrate buffer (pH 4) and added to the vine shoot flasks at a concentration of 16.7 U/g-biomass, resulting in a 5% w/v ratio. The flasks were closed with a plastic screw and incubated at 37 ºC, 130 rpm for 120 min (optimized conditions by Rabelo et al., 2022). All slurry resulting from enzymatic hydrolysis (solid plus liquid) was used in the fermentation assays. A condition without vine shoots was performed as a control of the biological pre-treatment to verify the contribution of the enzyme as a substrate in the fermentation assays.

Enzymatic hydrolysis with enzyme cocktail of cellulase, β-glucosidase, and hemicellulase (Cellic® CTec2, Novozymes A/S, Bagsværd, Denmark) was tested in addition to the pre-treatments. The wet solid obtained in SE, the solid obtained in OS, as well as the slurry from LAC pre-treatment, were used as substrate for enzymatic hydrolysis. Briefly, each solid (5% w/v, in dry weight) was mixed with 0.05 M citric citrate buffer (pH 4.8) and the enzyme cocktail (15 FPU/g-dry biomass). For the biological pre-treatment slurry, the pH of the liquid was changed to 4.8 with the addition of sodium citrate, and the enzyme cocktail (15 FPU/g-dry biomass) was added. The enzymatic hydrolysis flasks were kept at 50 °C, 150 rpm for 72 h (Padilla-Rascón et al., 2022). For comparison purposes, enzymatic hydrolysis was also carried out on raw material. The hydrolysis efficiency was determined from glucose concentration values obtained from enzymatic hydrolysis and the glucose potential concentration in the vine shoots (pre-treated or raw material).

Fig. 1 illustrates a scheme of the strategies for the use of vine shoots as a substrate in the fermentative H_2 production.

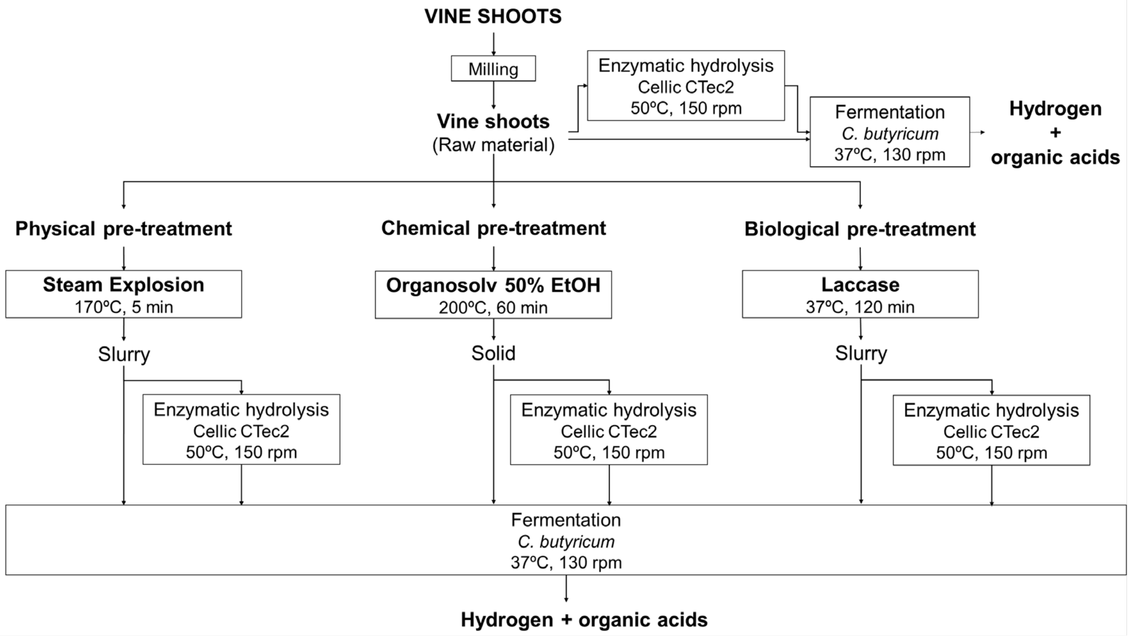

Fig. 1 Scheme summarizing the strategy used for the valorization of vine shoots for H_2 and organic acids production

2.3. Fermentative inoculum

Clostridium butyricum was used as a fermentation inoculum in the H_2 production assays. The pure culture was obtained from the Tropical Culture Collection (CCT7470) of the André Tosello Foundation (Campinas SP, Brazil). Cells were reactivated in Reinforced Clostridial Medium, according to the protocol provided by the Foundation, following the steps detailed in Moura et al. (2020).

After 36h of growth, aliquots corresponding to 5.7 g-TVS/L were centrifuged (1717 g-force/4 min), the supernatant was discarded and the pellet stored at -20 ºC.

2.4. Fermentative assays

Fermentation assays for H_2 production were carried out in the same flasks as in the enzymatic hydrolysis and/or pre-treatment step. The substrate assays were: vine shoots (raw material), steam explosion pre-treated vine shoots (SE vine shoots), organsolv pre-treated vine shoots (OS vine shoots) and laccase pre-treated vine shoots (LAC vine shoots), in addition to pre-treated plus hydrolyzed vine shoots (hydrolyzed raw material vine shoots, hydrolyzed SE vine shoots, hydrolyzed OS vine shoots and hydrolyzed LAC vine shoots).

A previously autoclaved macronutrient solution (5.0 g/L meat extract, 1.5 g/L yeast extract, 5.0 g/L peptone, 2.5 g/L NaCl and 1.5 g/L sodium acetate) was added to the flasks, the pH was adjusted to 6.8 with NaOH, and the fermentative inoculum was added last. The flasks (Isolab® flat bottom flask 100 mL, 50% reactional volume and 50% headspace) were then subjected to an atmosphere of N_2 (100%) for 10 min, closed with a silicone cap and plastic screw and incubated in an orbital shaker incubator at 37 ºC, 130 rpm.

Fermentation control assays were conducted under the same conditions as described, but (1) without vine shoots, (2) with laccase and without vine shoots, and (3) with cellulase and without vine shoots, to verify whether the H_2 production can only be derived by the macronutrients and/or enzymes addition.

2.5. Analytical methods

The determination of total volatile solids (TVS) of inoculum was performed according to APHA (2012). Analysis of the production of soluble metabolites (volatile fatty acids and monomeric carbohydrates) were determined by high performance liquid chromatography (HPLC) (Padilla-Rascón et al., 2022).

Cellulose, hemicellulose and lignin in the raw material and recovered solid of pre-treatment were carried out by gravimetric analysis in accordance with the protocol of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012) and reported as percentage of solid (g/100 g of raw material or g/100 g of pre-treated recovered-solids). The values of solids recovery percentages after the SE, OS and LAC pre-treatments (97.2, 53.1 and 88.4%, respectively) were considered for the comparative calculations of the pre-treated recovered-solids with the raw material.

The H_2 headspace gas was determined by gas chromatography (GC 2010 Shimadzu® Corp., Kyoto, Japan) equipped with a Carboxen column (1010 PLOT 30 m x 0.32 mm and 0.3 µm film thickness), a thermal conductivity detector (TCD) and argon as the carrier gas and PAL® autosampler (Zwingen, Switzerland). The injector temperature was 90 °C, in split mode (1:20) for 2 min. The injection volume was 40 µL. The oven temperature was 130 °C for 4.2 min. A ramp of 30 °C/min until 135 °C, maintained for 2 min. The column flow was 1.2 mL/min. The detector was maintained at 180 °C, employing at a current of 20 mA and a negative polarity.

Gaseous samples (1.0 mL) were collected directly from the headspace of the reactors using a syringe with a push button valve and transferred to 2 mL vials with vacuum. The vials were placed in the autosampler of the GC for analysis.

The H_2 data were adjusted to the modified Gompertz model (Zwietering et al., 1990) which has been described as a suitable model for the adjustment of accumulated biogas production data in batch experiments. The model calculates the H_2 production potential (P) based on the sigmoid curve obtained according to the H_2 concentration versus time. In general, when the model is well adjusted (with R^2 close to 1) P is always very close to the maximum value obtained experimentally. According to the model (Equation 1), it is possible to estimate the maximum H_2 production potential (P), maximum H_2 production rate (Rm), and time of initial H_2 production (λ):

$$\boldsymbol{H = P \cdot \exp\left\{- \exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}} \quad (1)$$

where H is the cumulative H_2 production per unit of headspace volume (mL/L), t is the operation time (h) and e is Euler number (2.71828). The parameters were calculated using OriginPro 9.0 software (OriginLab Corp., Northampton, MA, USA).

The H_2 production values obtained from biomass with the different pre-treatments were statistically compared using Student's t distribution (two tailed, for paired samples). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Structural analysis of pre-treated vine shoots

In the composition of the vine shoots, their content in structural carbohydrates stands out, mainly glucose (37.27%) and xylose (18.00%) which are the main constituents of the polysaccharides cellulose and hemicellulose, respectively. The complete chemical characterization of the raw vine shoots is presented in Table S1 (supplementary material).

According to the gravimetric analysis, the structural composition of vine shoots changed according to the applied pre-treatment (Fig. 2). Vine shoots raw material have a significant polysaccharide content (33.9% cellulose and 18.5% hemicellulose) in addition to 23.9% lignin. Due to this fermentable sugar content (52.4%), vine shoots are potential substrates in fermentative processes for the production of ethanol (Dávila et al., 2019; Senila et al., 2020) and methane (Pérez-Rodríguez et al., 2016). In order to compare the compositions of raw material and pre-treated recovered-solid, the structural composition of pre-treated vine shoots was expressed as a percentage in relation to the total solid content (and not in relation to the raw material biomass).

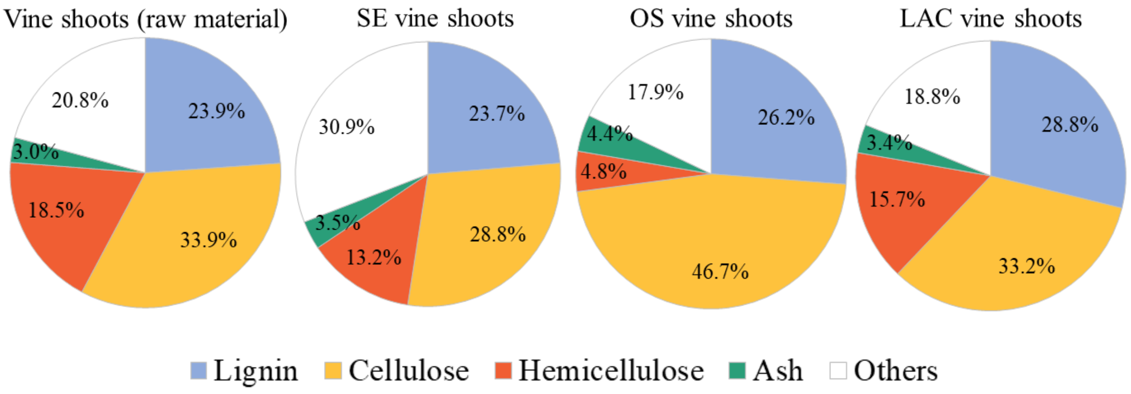

Fig 2. Composition of raw material and steam explosion (SE), organosolv (OS) and laccase (LAC) pre-treated recovered-solids. Values are expressed as a percentage of dry weight.

A slight decrease in the contents of structural polysaccharides (28.8% cellulose and 13.2% hemicellulose) was observed with the physical SE pre-treatment, probably resulting from their solubilization due to the low severity conditions of the SE system (Jacquet et al., 2011). In addition, a similar proportion of lignin was obtained in the pre-treated recovered-solids (23.7%).

With the addition of organosolvent in the chemical pre-treatment (OS), there was a marked decrease in the hemicellulose content (4.8%) and an increase in the cellulose content in the biomass (46.7%). The main objective of OS pre-treatment is the solubilization of lignin from biomass, along with hemicellulose. As a result, a significant increase in cellulose content is observed. As in the present study, Padilla et al. (2022) observed a significant increase in cellulose content (from 21.0 to 31.7%), hemicellulose solubilization (from 24.8 to 7.3%) and an increase in lignin content (from 32.9 to 49.5%) after organosolv pre-treatment of olive stones. The increase in lignin content observed in OS biomass is related to the decrease in hemicellulose content (in %) of the cell fiber and not to its quantitative increase.

After the biological LAC pre-treatment, few alterations were observed in the fiber composition of the biomass, since the contents of the LAC vine shoots (33.2% cellulose, 15.7% hemicellulose and 28.8% lignin) were close to those of raw material biomass (33.9% cellulose, 18.5% hemicellulose and 23.9% lignin). Similar results were obtained in the study conducted by Suman et al. (2022) with biomass of jute rods. The composition of biomass pre-treated with laccase (45.9% cellulose, 8.5% hemicellulose and 25.8% lignin) was similar to that of raw material biomass (44.9% cellulose, 9.7% hemicellulose and 26.1% lignin). However, the authors observed a difference in the spectra of treated and untreated samples by Fourier transform infrared spectroscopy analysis. There was a decrease in the OH functional group induced by the formation of active radicals due to electron delocalization, in addition to an increase in free CH_2 groups indicating ring opening in lignin compounds. Furthermore, according to porosity studies carried out by Rajak et al. (2018), after enzymatic pre-treatment of kans grass biomass, changes in biomass properties are directly related to the action of enzymes, since the authors observed changes in surface area, decrease in pore size and pore volume of enzymatically pre-treated biomass. Thus, in the LAC pre-treatment of vine shoots, although no changes were observed in the gravimetric analysis, changes in the lignin functional groups and changes in properties of the biomass may have occurred due to enzymatic pre-treatment.

Random laccase attack forms radical products that can undergo further oxidation or undergo other reactions such as hydration, disproportionation, or polymerization reactions (Gonzalo et al., 2016). Chen et al. (2012) observed the degradation and depolymerization of lignin in laccase-treated ensiled corn stover by tetramethylammonium hydroxide thermochemolysis and gas chromatography–mass spectroscopy analysis. Molecular signals of lignin decomposition were observed by the increasing ratios of acids to aldehydes, demonstrating evidence that the laccase system induces significant oxidation of the lignin side chain.

Alterations in the cell wall structure were also examined by polarized optical microscopy, which displayed the scattered cellulose building block (microfibrils) in wheat straw pre-treated by laccase, compared with the tightly arranged microfibrils in untreated control straw (Lu et al., 2010). In sugarcane bagasse pre-treated with laccase, fibers with a more irregular structure and more indentations were also observed by scanning electron microscopy (Rabelo et al., 2022). Therefore, in the vine shoots pre-treatment, although a decrease in lignin content was not quantified in gravimetric analysis, the pre-treatment by laccase may have caused re-localization of lignin (Masran et al., 2016), causing a structure modification, rather than lignin removal, which also contributes to the digestibility and accessibility of the cellulose for hydrolysis and fermentation (Kristensen et al., 2008; Lu et al., 2010).

3.2. Effect of vine shoots pre-treatments on H_2 production

The relation between the biomass pre-treatment and the increase in fermentative products is an indirect approach. With pre-treatment, whether physical, chemical or biological, the degree of biomass polymerization is reduced, which provides an increase in accessible surface area for the cellulose hydrolysis (Zhang et al., 2023). As a result, there is an increase in the concentration of soluble sugars that will be converted in greater amounts into H_2 (Rabelo et al., 2022), ethanol (Fang et al., 2015) and/or volatile fatty acids (VFA) (Rabelo et al., 2018).

The H_2 production potential increased from pre-treated vine shoots (649.4, 399.8, and 749.7 mL/L for SE, OS, and LAC pre-treatments, respectively) relative to raw material biomass (300.1 mL/L) (Fig. 3A).

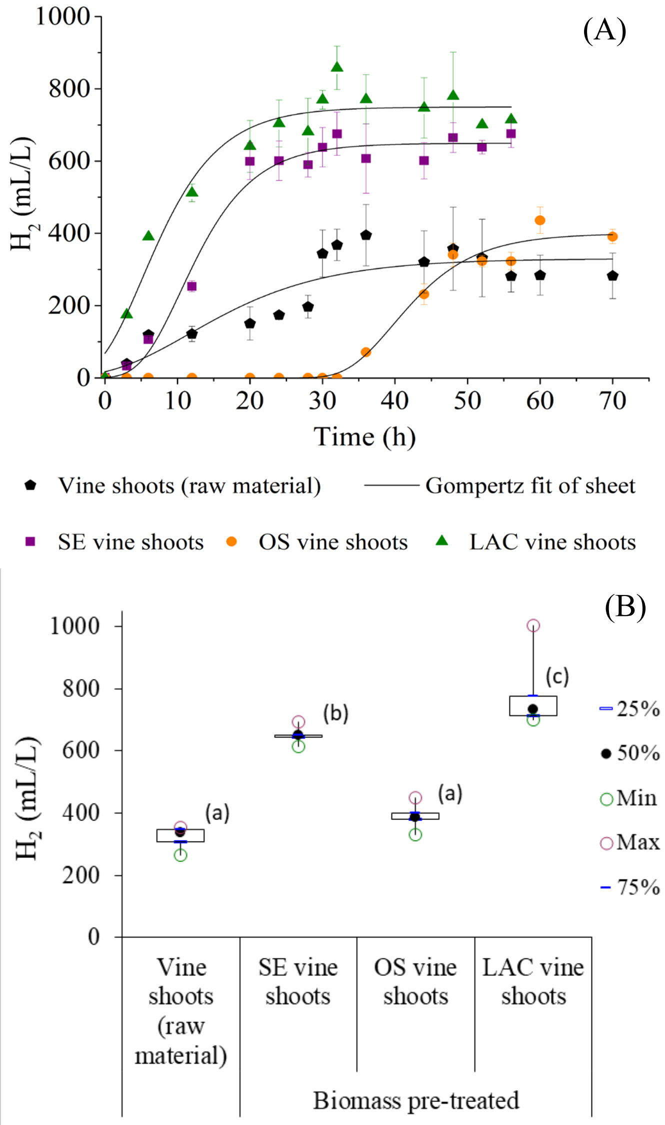

Fig 3. (A): H_2 production from raw material and pre-treated vine shoots by steam explosion (ES), organosolv (OS), and laccase (LAC). (B): Boxplots of H_2 production (mL/L) from raw material and pre-treated vine shoots. Different lowercase letters indicate significant differences at the $p < 0.05$ level (n = 5).

A hydrolysis of glycosidic bonds in the hemicelluloses and, to a lesser extent, in the cellulose of biomass are achieved in the physical pre-treatment by steam explosion. A cleavage of hemicellulose-lignin bonds can also occur. Thereby, cellulose with a reduced degree of polymerization (DP) is obtained in the solid residue (Li et al., 2007).

In the case of sugarcane straw, 92.7% of hemicellulose solubilization was obtained with steam explosion (200 ºC/15 min), but it required an alkaline pre-treatment (NaOH, 100 ºC/1 h) to achieve lignin solubilization (81.3%) (Oliveira et al., 2013). Wanderley et al. (2013) also evaluated alkaline delignification (NaOH, 100 ºC/1 h) before steam explosion pre-treatment (200 ºC/7 min) of sugarcane bagasse. The authors reported a decrease from 25.8% to 8.9% in hemicellulose content after steam explosion, and no removal of cellulose and lignin. 84% lignin removal was achieved with the previous alkaline delignification. Hydrolysis and fermentation of the solid pre-treated with NaOH and steam explosion resulted in a 450% increase in ethanol production (23.38 g/L) compared with the solid pre-treated with steam explosion alone (5.21 g/L) (Wanderley et al., 2013).

In the present study, a previous chemical treatment was not carried out before the steam explosion so as to have no separation of solid and liquid phases and thus use all the slurry for fermentation. Therefore, as expected for biomass pre-treated by steam explosion (with recuperation of 97.2%), a decrease in pre-treated recovered-solid was observed with hemicellulose (30.6%) and cellulose (17.5%) contents, but not in the lignin content, in reference to raw material. These changes in the structure of the vine shoots due to the steam explosion were favorable to the fermentation of the biomass, since more than 216.4% of H_2 was produced (649.4 mL/L) than raw material vine shoots (300.1 mL/L).

Organosolv pre-treatment has been evaluated as an effective method for high-lignin lignocellulosic biomass such as olive stones (lignin, 35.6%) (Padilla-Rascón et al., 2022), sugarcane bagasse (lignin, 27.2%) (Mesa et al., 2010) and empty fruit bunches (lignin, 24.0%) (Alriols et al., 2009). In the case of vine shoots (lignin, 23.9%), due to the pre-treatment conditions (organic solvent at high temperatures), in addition to the solubilization of lignin (43.6% in relation to raw material biomass), there was also solubilization of hemicellulose (86.1%) and cellulose (26.8%), with 53.1% solid recuperation. However, the changes in the cell fiber were not enough to increase the bacterial hydrolysis/fermentation, since the 133.2% more H_2 produced from the OS vine shoots (399.8 mL/L) were not statistically significant in relation to raw material biomass (300.1 mL/L) (Fig. 3B).

The following aspects were observed regarding the use of OS vine shoots: The production of H_2 took longer to start (λ, 39.7h for OS vine shoots, while 11.6h for raw material vine shoots, 10.4h for SE vine shoots and 5.2h for LAC vine shoots), this was probably given the need for an additional step of bacterial hydrolysis of cellulose, and a lower amount of H_2 produced (399.8 mL/L) in relation to the other pre-treated biomass, probably due to the loss of readily available sugars for the liquid fraction. The purpose of laccase pre-treatment is also the biomass delignification. However, no decrease in LAC vine shoots lignin content was observed after laccase pre-treatment. Laccase has already been applied to different types of biomass, such as sugarcane bagasse (Dionizio et al., 2022), wheat straw (Moreno et al., 2012), sweet sorghum straw (Shanmugam et al., 2018) and corn stover (Fang et al., 2015) for delignification, which resulted in increased production of H_2 (Dionizio et al., 2022) or ethanol (Fang et al., 2015). In the present study, laccase was applied for the first time in vine shoots, and there was a significant increase in the amount of H_2 produced (from 300.1 to 749.7 mL/L). In the control assay (with laccase and without vine shoots) no H_2 was produced.

The greatest increase in the amount of H_2 was obtained with the vine shoots pre-treated (249.8, 216.4 and 133.2% with LAC, SE and OS, respectively), in relation to the raw material biomass. The disruption of the vine shoots fibers by pre-treatments is essential to increase the production of biofuels, as well as other biomass consolidated as substrates in fermentative processes (Table 1).

444 **Table 1:** Pre-treated biomass used as a substrate for biofuels production

Biomass	Pre-treatment Physical (P), Chemical (C) or Biological (B)	Goal of pre-treatment	Increase in the biomass conversion into biofuels	Reference
Pulp and paper biosludge	(B) Protease	Degradation of the extracellular polymeric substances matrix	Biogas 26%	Bonilla et al., 2018
Food waste	(P) Mechanical pre-treatment	Increased surface area available to microorganisms	CH_4 28%	Izumi et al., 2010
Corn stover	(B) Cellulase	Disruption of surface structure and degradation of cellulose	Biogas 36.9%	Wang et al., 2018
Barley straw and wheat straw	(P) Mechanical pre-treatment	It does not produce inhibitory or toxic substances	CH_4 54.2% and 83.5%	Menardo et al., 2012
Corn stover	(C/F/B) H_2SO_4 + steam explosion + laccase	Removes a small part of the lignin, depolymerizes cellulose and hemicellulose	Ethanol 111%	Fang et al., 2015
Paper industry wastes	(C) H_2SO_4	Simplicity, no special equipment required, and less time consuming	H_2 179%	Moreno-Dávila et al., 2017
Sugarcane bagasse	(B) Laccase	Alteration of the lignocellulosic matrix	H_2 184%	Rabelo et al., 2022
Sida hermaphrodita and *Miscanthus giganteus*	(C) NaOH	High delignification level and significant hemicellulose degradation	CH_4 146.7% and 194.7%	Michalska et al., 2015
Sugarcane bagasse	(B) Laccase	Improved delignification, maximizing biomass fermentation	H_2 196.1%	Dionizio et al., 2020
Sugarcane bagasse	(C/F) H_2O_2 assisted hydrothermal	Residue rich in cellulose content	CH_4 218.7%	Ahmad et al., 2018
Sweet sorghum stover	(B) Laccase	Delignification in an eco-friendly and precise manner	H_2 326%	Shanmugam et al., 2018
Vine shoots	(F) Steam explosion	Solubilization of cellulose and hemicellulose	H_2 216.4%	**Present study**
Vine shoots	(C) Organosolv	Biomass delignification	H_2 133.2%	**Present study**
Vine shoots	(B) Laccase	Structural changes in cell fiber	H_2 249.8%	**Present study**

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3.3. Effect of enzymatic hydrolysis of vine shoots pre-treated on H_2 production

The pre-treated vine shoots were submitted to enzymatic hydrolysis by a cellulase complex and there was saccharification of the substrate into glucose. Saccharification efficiency varied according to the type of vine shoots pre-treatment, from 3.5% for raw material biomass, 7.3% for LAC vine shoots, 53.1% for SE vine shoots and 77.0% for OS vine shoots (Table S2).

The use of laccase as a pre-treatment of vine shoots increased the amount of glucose converted from biomass by 184% (from 1.3 to 2.4 g/100g vine shoots). However, the efficiency of the enzymatic hydrolysis of LAC vine shoots was low (7.3%) compared with other pre-treated biomass (53.1% with SE vine shoots and 77.0% with OS vine shoots). The increase in the concentration of soluble sugars as a result of biomass pre-treatment by laccase has been reported before (Rabelo et al., 2022; Sherpa et al., 2018), and is related to the deconstruction of lignin and exposure of hemicellulose and amorphous regions of cellulose, resulting in the release of sugars that were entangled in the cellular fiber, to the liquid medium. These modifications in biomass structure can enhance bacterial hydrolysis that precedes fermentation of lignocellulosic materials (Dionizio et al., 2022), such as vine shoots. Therefore, even with some initial amount of soluble sugars present, an increase in soluble sugars may result from the hydrolysis of cellulose and hemicellulose. Monitoring the concentration of soluble sugars throughout the incubation period of batch reactors was one of the objectives of the work of Rabelo et al. (2018) during the fermentation of sugarcane bagasse in H_2. The authors confirmed the concomitant increase and decrease of sugar concentration during fermentation. Accordingly, in the present study, the initial amount of soluble sugars reported in Table 2 may have increased over time due to bacterial hydrolysis of vine shoots, making it impractical to calculate the H_2 yield based on the molar ratio between the H_2 produced and the glucose initially present. Thus, even with low enzymatic yield, structural changes and depolymerization of LAC vine shoots were favorable for hydrolysis and fermentation, since 184% more soluble sugars were solubilized from the biomass and 213.3% more H_2 was produced (1252.9 mL/L), in relation to the hydrolyzed raw material biomass (587.4 mL/L).

According to Ruiz et al. (2008), the efficiency of enzymatic hydrolysis of pre-treated steam explosion biomass improved with increasing pre-treatment temperature. At 220 ºC steam explosion of sunflower stalks, the hydrolysis efficiency was 72%. If, on the one hand, there is an increase in the efficiency of enzymatic hydrolysis due to the solubilization of the hemicellulose and an increase in the cellulose content of the fiber, on the other hand, the increase in temperature implies an increase in cost and an increase in the formation of lignin degradation products, which may be fermentation inhibitors and need to detoxify the pre-treated liquor (Romero-García et al., 2022). For the steam explosion of vine shoots, 170 ºC was used in the system and 53.1% of efficiency was achieved in the enzymatic hydrolysis, however there was no generation of products that inhibit fermentation, and all pre-treated slurry of SE pre-treatment (solid plus liquid) was used as substrate for fermentation without the need for an additional detoxification step.

The highest enzymatic efficiency was achieved with the OS vine shoots (77.0%), certainly a consequence of the solubilization of the lignin and hemicellulose components and greater cellulose recovery in biomass (46.7%) for enzymatic hydrolysis. H_2 production from hydrolyzed biomass increased proportionally to the enzymatic hydrolysis yields, with the highest production also with hydrolyzed OS vine shoots (2406.5 mL H_2/L) (Fig. 4). In the control assay (with cellulase and without vine shoots) no H_2 was produced.

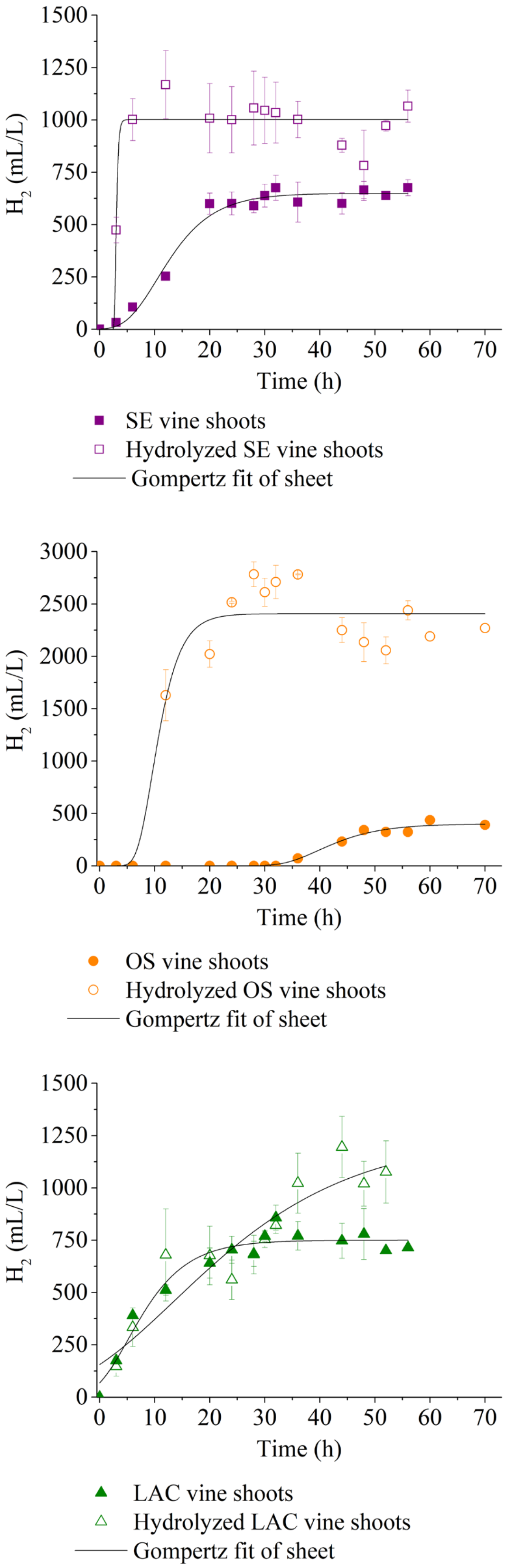

Fig 4. H_2 production from pre-treated vine shoots by steam explosion (SE), organosolv (OS) and laccase (LAC), and hydrolyzed pre-treated vine shoots.

The volume of H_2 produced increased in all fermentation assays in which hydrolyzed pre-treated vine shoots were used as substrate in relation to non-hydrolyzed biomass. An increase of 154.4 and 167.1% in the amount of H_2 using SE biomass and LAC biomass were produced with the respective hydrolyzed biomass (1001.2 and 1252.9 mL H_2/L, respectively) in relation to the non-hydrolyzed biomass (649.4 and 749.7 mL H_2/L, respectively). However, the sharp increase in H_2 production after hydrolysis was with fermentation of OS vine shoots (over 600%). With the hydrolyzed OS vine shoots 2406.5 mL/L were produced, compared with 399.8 mL/L with the non-hydrolyzed biomass.

All H_2 production results obtained from the vine shoots fermentation, adjusted according to the modified Gompertz model, are shown in Table S3.

In general, the fermentation of various types of hydrolyzed biomass produces greater amounts of H_2, due to the availability of readily fermentable sugars for conversion to H_2. Tang et al. (2013) studied fermentative H_2 production using different enzymatic hydrolysates pre-treated of corn cob, but obtained similar H_2 production independent of substrate pre-treatment. The authors reported 1215, 1184 and 1220 ml H_2/L when enzymatic hydrolyzed pre-treated with acid steam explosion, alkali soaking and steam explosion were used as substrate, respectively. Ren et al. (2010) reported the best performance of H_2 production (2430.4 mL/L) from hydrolyzed corn stover. The substrate underwent the first delignification with 2% NaOH and then hydrolyzed by cellulase and xylanase.

It is clearly observed that when using hydrolyzed vine shoots as substrate, the H_2 production (749.7 to 2406.5 mL/L) obtained from this study is comparable or higher than other hydrolyzed biomass already used as substrate (Table 2). Therefore, the H_2 production can also be one of the products with biotechnological interest obtained from vine shoots and/or hydrolyzed vine shoot.

Table 2: H_2 production from hydrolyzed biomass

Biomass	Pre-treatment	Hydrolysis	Total sugar (g/L) [a]	Glucose (g/L) [a]	Inoculum fermentative	H_2 (^{b})	H_2 (mL/L)	Reference
Corn stover	Alkali (NaOH)	Cellulase and xylanase	15.1	11.2	*Thermoanaerobacterium thermosaccharolyticum*	108.5 mmol/L	2430.4	Ren et al., 2010
Rice mill wastewater	Thermic (Autoclave)	*Aspergillus niger* enzymes	15.8	12.3	*E. aerogenes*	3480 mL	6960	Ramprakash and Muthukumar, 2014
Apple pomace	Acid (HCl)	Cellulase	-	-	River sludge	134.04 mL/g TS	178.7	Wang et al., 2010
Sugarcane bagasse	-	Acid hydrolysis	11.3	1.5	Elephant dung	1314.08 mL/L	1314.1	Fangkum and Reungsang, 2011
Waste paper	Acid (H_2SO_4)	Acid hydrolysis	18.9	-	Anaerobic sludge	125.9 mL	1258.9	Eker and Sarp, 2016
Corn cob	Acid steam explosion	Cellulase	5.1	3.3	*C. hydrogeniproducens*	1215 mL/L	1215	Tang et al., 2013
Corn cob	Alkali soaking	Cellulase	6.1	3.6	*C. hydrogeniproducens*	1184 mL/L	1184	Tang et al., 2013
Corn cob	Steam explosion	Cellulase	4.7	2.8	*C. hydrogeniproducens*	1220 mL/L	1220	Tang et al., 2013
Wheat flour	-	Glucoamylase	-	35.1	Anaerobic sludge	2379.6 ml	1586.4	Han et al., 2015
Corn stover	Washing and grinding	Acid hydrolysis	5	0.9	Anaerobic granular sludge	821.8 mL/L	821.8	Zhang et al., 2015
Vine shoots	Laccase	Cellulase	1.9	1.3	*C. butyricum*	1252.9 mL/L	1252.9	**Present study**
Vine shoots	Steam explosion	Cellulase	7.2	5.7	*C. butyricum*	1001.2 mL/L	1001.2	**Present study**
Vine shoots	Organosolv	Cellulase	25.9	22.0	*C. butyricum*	2406.5 mL/L	2406.5	**Present study**

[a] In hydrolysate, after hydrolysis

[b] Unit reported by the author

3.4. Metabolites redistribution during fermentation of vine shoots and hydrolyzed vine shoots

During the hydrolyzed vine shoots fermentation, H_2 production was accompanied by production of metabolic products, mainly butyric acid and acetic acid, typical mixed acid fermentation pathway of *Clostridium butyricum* (Rainey, 2009). As in other works (Gupta et al., 2014; Rabelo et al., 2020), the substrate type regulated the formation of the final product (acetic or butyric acid). Rabelo et al. (2020) observed greater production of acetic acid (2.9 g/L) with glucose fermentation, and greater production of butyric acid (2.8 g/L) with cellulose fermentation by *Paraclostridium* sp. The metabolic pathway of *C. butyricum* was altered due to the type of pre-treated biomass, since acetic acid was the main metabolite observed with hydrolyzed SE vine shoots and hydrolyzed LAC vine shoots (3.2 and 9.7 g/L, respectively), and butyric acid was the main acid observed in hydrolyzed OS vine shoots fermentation (11.4 g/L).

The mixed acid pathway also occurred in the fermentation of pre-treated vine shoots, but like the H_2 production, the production of soluble metabolites was lower than in the fermentation of hydrolyzed biomass. In SE vine shoots and LAC vine shoots, acetic acid was the main metabolite observed (2.7 and 9.0 g/L, respectively), while in OS vine shoots, butyric acid was the most produced (9.7 g/L) (Fig. S1).

The fermentative production of butyric acid from renewable raw materials has been widely studied due to increasing demands in the pharmaceutical and food industries (Fu et al., 2022) and environmental and sustainable concerns with its chemical production (Oh et al., 2019). Butyric acid production (5.8 g/L) from sugarcane bagasse was reported by Rabelo et al. (2020) during the H_2 production with *Paraclostridium* as inoculum and by Dionizio et al. (2022) with *C. butyricum* (4.7 g butyric acid/L). However, those previous reports, like the present study, did not focus on the optimization of butyric acid production, but on the H_2 production.

Butyric acid production values are higher when the study objective is the effective production of this acid. To increase the production of butyric acid, Chi et al. (2018) added C. *tyrobutyricum* as inoculum and obtained an increase from 5.53 to 18.05 g/L with the culture addition. Oh et al. (2019) demonstrated that mixed biomass with alga and rice straw was effective as substrate in butyric acid production (14.77 g/L) with *C. tyrobutyricum*. Fu et al. (2022) determined optimal fermentation parameters (37 ºC, 5 g/L of yeast extract, pH 6.0 and 80 g/L of substrate) for the production of butyric acid (21.72 g/L) from raw cassava starch and *C. butyricum* SCUT620 as inoculum.

In the present study, the redistribution of metabolites was directly related to the pre-treatment strategies, obtaining the highest butyric acid production of 11.4 g/L with OS hydrolyzed vine shoots. In this sense, the increase of butyric acid production is another possibility of using vine shoots in fermentation processes, especially with the biomass pretreated by organosolvent.

The mass balance and the obtained yields from vine shoots fermentation are shown in detail in Fig. 5. The conditions that include the use of LAC vine shoots and hydrolyzed OS vine shoots were considered for the mass balance, since the highest yields of fermentative products (H_2 and organic acids) were achieved. For comparative purposes, the yield considered the recovery of the material after the respective pre-treatments (88.4% for laccase and 53.1% for organosolv) and were calculated from 100 kg of raw material.

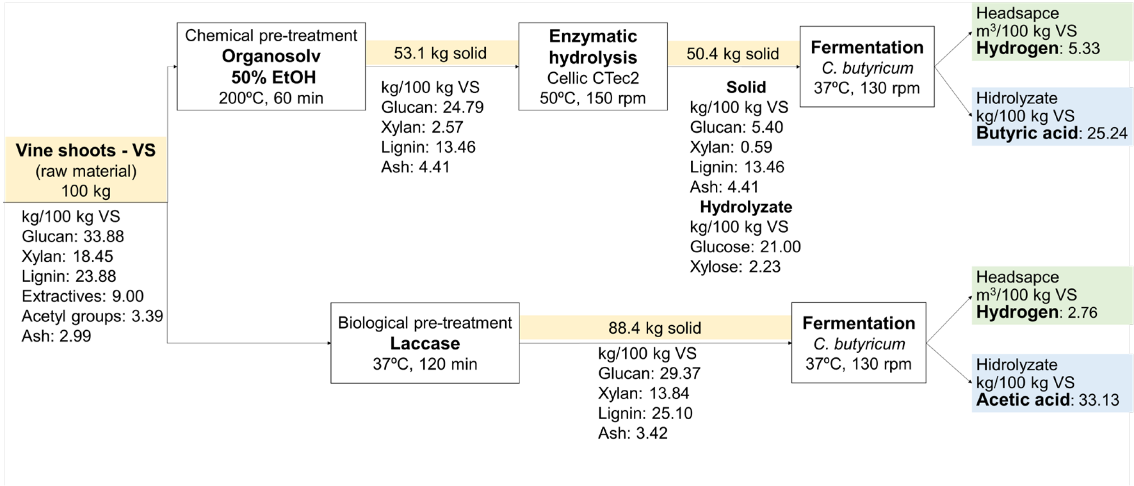

Fig 5: Mass balance obtained through the fermentation from hydrolyzed pre-treated organosolv vine shoots and pre-treated laccase vine shoots

4. Conclusion

The strategy proposed in this work allows, for the first time, the valorization of vine shoots in fermentative processes for the production of biological H_2 and also organic acids (acetic and butyric). The amount of H_2 produced increased by 216.4, 133.2 and 249.8% after physical, chemical and biological pre-treatments of vine shoots, in relation to *in nature* biomass. With hydrolysis of pre-treated biomass, H_2 production was even higher (1001.2, 2406.5 and 1252.9 mL H_2/L with hydrolyzed SE, OS and LAC vine shoots, respectively). Therefore, in order to obtain higher H_2 production from vine shoots, pre-treatment with laccase was the most effective (749.7 mL/L). If one considers the addition of the enzymatic hydrolysis step, organosolv was the most effective (2406.5

mL/L). The mixed acid pathway was predominant in all conditions, since acetic and butyric acids were produced. The maximum value of butyric acid was 11.4 g/L with hydrolyzed OS vine shoots.

There are several significant points in the overall process that can be exploited and optimized to increase the H_2 and/or soluble metabolites production. In fact, vine shoots can be used as substrates in fermentation processes, and the importance of pre-treatment of the biomass was verified. Thus, from this first study that addresses the H_2 production from vine shoots, the optimization of parameters (namely, solid-liquid ratio, evaluation of severity of the SE process, reduction/reuse of liquid waste in OS pre-treatment, optimization of laccase addition for biological pre-treatment) will be the continuation of experimental work with vine shoots to increase H_2 production before important scale-up and technical-economic and environmental evaluations.

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.

Acknowledgments

The authors want to acknowledge the financial support from São Paulo Research Foundation (FAPESP) (Processes numbers: 2018/26470-7 and BEPE 2020/04000-9) and MCIN/AEI/10.13039/501100011033/FEDER, reference project PID2020-112594RB-C31. J.M. Romero-García expresses his gratitude to the Junta de Andalucía for financial support (Postdoctoral researcher R-29/12/2020).

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Supplementary material

Vine shoots pre-treatment strategies for improved hydrogen production and metabolites redistribution in *Clostridium butyricum*

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S1. Results and Discussion

Table S1 contains the chemical characterization of the raw vine shoots.

Table S1: Composition of raw material (%)

Composition	g/100 g vine shoots (dry basis) Mean values of three replicates ± standard deviation
Cellulose [a]	33.88 ± 1.04
Glucose	37.27 ± 0.24
Hemicellulose [b]	18.45 ± 0.43
Xylose	18.00 ± 0.32
Galactose	1.47 ± 0.11
Arabinose	0.81 ± 0.08
Mannose	0.52 ± 0.03
Total lignin	23.88 ± 0.53
Acid insoluble lignin (AIL)	22.11 ± 0.53
Acid soluble lignin (ASL)	1.77 ± 0.04
Ash	2.99 ± 0.03
Others	
Acetyl groups	3.39 ± 0.04
Galacturonic acid	1.06 ± 0.09
Extractives	9.00 ± 0.36
Glucose in extractives	0.77 ± 0.03

Mean values of three replicates ± SD

[a] as glucose

[b] as hemicellulose sugars

Saccharification efficiency varied according to the type of vine shoots pre-treatment (Table S2).

Table S2: Efficiency of saccharification of vine shoots

Vine shoots	g glucose/100 g vine shoots	Efficiency [a] (%)	Yield [b] (%)
Raw material	1.3	3.5	3.5
Pre-treated by laccase	2.4	7.3	6.3
Pre-treated steam explosion	11.5	53.1	31.0
Pre-treated organosolv	21.0	77.0	56.3

[a] in relation to theoretical of glucose in pre-treated material
[b] in relation to theoretical of glucose in raw material

All results obtained from the vine shoots fermentation, H_2 production (*P*), H_2 production rate (*Rm*) and H_2 production start time (λ), adjusted according to the modified Gompertz model, are shown in Table S3.

Table S3: H_2 production parameters adjusted by modified Gompertz model for vine shoots fermentation and in terms of productivity (m^3 H_2/100 kg biomass)

	P (mL/L)	*Rm* (mL/L.h)	λ (h)	R^2	m^3 H_2/100 kg biomass
Vine shoots (raw material)	300.12	0.09	11.59	0.77	1.3
SE vine shoots	649.4	0.18	10.43	0.98	2.7
Hydrolysed SE vine shoots	1001.2	3.78	2.92	0.9	4.2
OS vine shoots	399.77	0.16	39.73	0.98	1.7
Hydrolysed OS vine shoots	2406.5	0.36	9.57	0.93	10.0
LAC vine shoots	749.74	0.17	5.17	0.95	3.1
Hydrolysed LAC vine shoots	1252.9	0.05	13.6	0.86	5.2

SE: Steam explosion physical pre-treatment, OS: organosolv chemical pre-treatment, LAC: Laccase biological pre-treatment

Production of acetic and butyric acids by *Clostridium butyricum* during vine shoots fermentation is shown in Fig. S1.

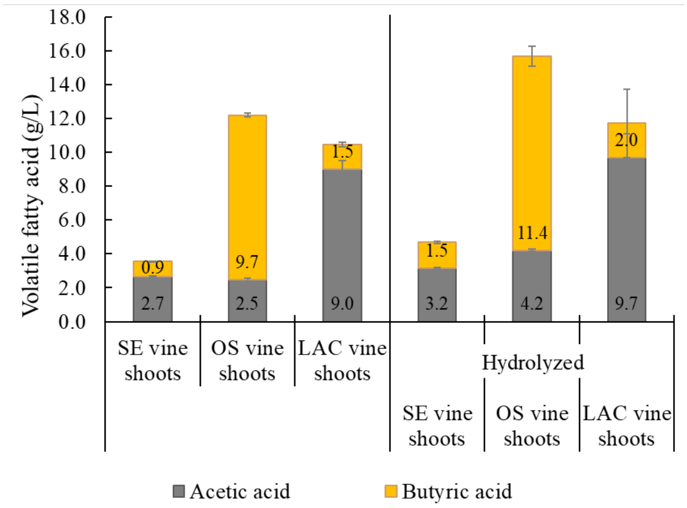

Fig. S1: Acetic and butyric acids produced during fermentation of pre-treated vine shoots and hydrolyzed pre-treated vine shoots.