



## Integral valorization of vine pruning residue by sequential autohydrolysis stages



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### ABSTRACT

Wine processing generates a large amount of residue, in particular pruning residue of vine. In this work, autohydrolysis in two sequential stages was proposed for the integral valorization of this residue. In a first stage, vine pruning residue was submitted to autohydrolysis treatment at 180 °C for 60 min (severity of 4.13) and liquid to solid ratio of 6 g water per g vine pruning residue. In these conditions, 63.7% of xylan was recovered in the liquid phase as xylooligosaccharides (17 g/L) and 2.35 g/L of phenolic compounds with antioxidant activity were also extracted. Autohydrolyzed vine pruning residue was subjected to a second autohydrolysis at temperature in the range 180–200 °C and time 30–40 min. After sequential treatments, enzymatic hydrolysis of cellulose was significantly improved from 73% to 99% of conversion. At selected conditions (severity of 4.60), ethanol production was successfully obtained from two strategies of, separately and simultaneously, saccharification and fermentation, thus achieving ethanol yield of 96 and 83%, respectively. Overall, two sequential stages of the process allowed the recovery of 13.7 kg of xylooligosaccharides, 3.1 kg of phenolic compounds, 13.1 kg of ethanol and 27 kg of lignin per 100 kg of vine pruning residue. Sequential autohydrolysis stages were shown as a suitable strategy for the integral valorization of vine pruning residue.

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## 1. Introduction

The bioeconomy is based on the optimal use of renewable resources (such as lignocellulosic biomass) through the development of biorefineries for the co-production of fuels and high-value bio-based products as chemicals and materials (Hennig et al., 2016; Karlsson et al., 2014). In particular, high-value bio-based products are obtained by a sustainable process based on principles of green chemistry (Peleteiro et al., 2014).

Lignocellulosic biomass includes wastes from agriculture, forestry and related industries (Ferreira et al., 2009). In Portugal, the wine industry generates during wine processing a lot of residues (such as vine pruning residue, VPR) with an estimated annual production of 1.2–3.5 t/ha (Brito et al., 2014). These residues are typically left in the agricultural field, used as domestic fuel due to calorific power or burnt in the field which causes environmental

pollution (Devesa-Rey et al., 2010). The potential energy that these residues could provide has been estimated in 190 GW per year or alcohol equivalent of 357 million liters (Ferreira et al., 2009). The Portuguese experience is limited to combustion of biomass. Nevertheless, VPR is a lignocellulose biomass enriched with bio-based compounds with potential to be transformed into commercial products by selective fractionation of the main components (cellulose, hemicellulose and lignin) (Dávila et al., 2016). Therefore, the valorization of these residues under a biorefinery approach could contribute attaining an energy and rural sustainable development (Lin et al., 2014).

Several pretreatments have been proposed for the processing of vine pruning residue, such as alkali pretreatment for glucose production (Cotana et al., 2015), enzymatic hydrolysis and ultrasounds pretreatments to obtain methane and biogas (Pérez-Rodríguez et al., 2016), or ethylene glycol pulping for paper sheets manufacturing (Jiménez et al., 2008). Recently, hydrothermal treatment (known as autohydrolysis) has been used for xylooligosaccharides (Dávila et al., 2016) and ethanol production (Buratti et al., 2015). Moreover, this residue is enriched in polyphenolic compounds (Max et al., 2010; Pérez-Rodríguez et al., 2016) with potential antioxidant features. Autohydrolysis has also been

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## Nomenclature

### Abbreviations

ABTS	2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ATR	Attenuated total reflectance
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DTG	Derivative Thermogravimetric
FPU	Filter Paper Units
FT-IR	Fourier-transform infrared
GAE	Gallic Acid Equivalent
HMF	Hydroxymethylfurfural
HPLC	High-performance Liquid Chromatography
SEM	Scanning Electron Microscopy
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
TGA	Thermogravimetric Analysis
UHPLC	Ultra-high-performance liquid chromatography
UI	Unit International
VPR	Vine Pruning Residue

### Parameters and constants

$\Omega$	Empirical parameter (14.75 K)
$A_0$	Absorbance of control (DPPH or ABTS)
$A_1$	Absorbance of autohydrolysis liquor
$B$	Biomass concentration (g/L)
$C$	Cellobiose concentration (g/L)

$E_a$	Activation energy (J/mol)
ESR	Enzyme to solid ratio (FPU per g)
$EtOH_0$	Ethanol concentration at the beginning of the fermentation (g/L)
$EtOH_f$	Ethanol concentration at the end of the fermentation (g/L)
$F$	Glucan fraction in dry biomass (g per g)
$G$	Glucose concentration (g/L)
GGC	Glucan to glucose conversion (%)
$I_a$	Inhibition activity (%)
$IC_{50}$	Concentration of sample or standard that can inhibit 50% of DPPH or ABTS (mg/mL)
LSR	Liquid to solid ratio (g per g)
$R$	Ideal gas constant (J/mol K)
$R_0$	Reaction ordinate (min)
$S_0$	Severity (dimensionless)
SY	Solid yield (g autohydrolyzed VPR per 100 g of VPR, oven dry basis)
$T_{(t)}$	Represent the temperature profile in the heating stage (K)
$T'_{(t)}$	Represent the temperature profile in the cooling stage (K)
$t_F$	Time needed for the whole heating–cooling period (min)
$T_{MAX}$	Target temperature (K)
$t_{MAX}$	Time needed to achieve the target temperature (min)
$T_{REF}$	Reference temperature (373.15 K)
$Y_{Et}$	Ethanol yield (%)

proposed for the extraction of phenolic compounds from lignocellulosic biomass (Egüés et al., 2012). Therefore, autohydrolysis is considered a green and sustainable technology suitable to be used in lignocellulosic biorefineries (Gullón et al., 2012). Yet, an integrated approach for the valorization of whole biomass fractions is mandatory to achieve a cost-competitive process (Singhvi et al., 2014). In this sense, one of the autohydrolysis limitations is the difficulty to attain an optimal condition for oligosaccharides manufacturing and suitable saccharification of cellulose into glucose for fuel production (Romaní et al., 2010). Typically, cellulose is more prone to saccharification after harsh conditions of pretreatment while in these severe conditions the xylose and xyloligosaccharides are degraded into sugars and/or inhibitors compounds such as furfural (Guilliams et al., 2016). Thus, the strategy employed for this two-target goal usually includes the use of two treatments (such as an autohydrolysis combined with a delignification process) (Romaní et al., 2011, 2016). Delignification processes solubilize the lignin and, consequently, the solid fraction is enriched in glucan being more susceptible to enzymatic hydrolysis (Romaní et al., 2011). Nevertheless, delignification treatments use alkalis and organic solvents, demanding additional steps of washing and neutralization. Moreover, the handling of these chemicals is less safe (Chaturvedi and Verma, 2013). As alternative to delignification process, autohydrolysis in two sequential stages at milder conditions (with water as only catalyst) is shown as an attractive and cleaner solution to disrupt lignocellulosic matrix attaining a suitable recovery of all its components or derivatives (Lee et al., 2010; Min et al., 2015).

In this study, a process using autohydrolysis in two sequential stages was used for the fractionation and integral valorization of VPR. The conditions of operation (temperature and time, severity in the range of 3.36–4.90) were evaluated in order to maximize the

hemicellulose-derived compounds recovery (as oligosaccharides) and for quantification and identification of phenolic compounds in autohydrolysis liquor. Moreover, an alternative second autohydrolysis of pretreated solid from the first autohydrolysis was carried out (severity in the range of 4.36–4.69) to improve the enzymatic accessibility of cellulose for glucose production and further fermentation into ethanol by simultaneous and separate saccharification and fermentation (SSF and SHF, respectively).

## 2. Materials and methods

### 2.1. Raw material and analysis of chemical composition

Vine pruning residue was provided from a local producer from Minho region, Northern Portugal. VPR was air-dried, milled to pass an 8 mm screen, homogenized in a single lot to avoid differences in the composition, and stored at room temperature in a dark and dry place until use. The raw material was analyzed by TAPPI (Technical Association of the Pulp and Paper Industry) standards for extractives, moisture, ashes and quantitative acid hydrolysis with 72% (w/w) sulphuric acid (T-264-cm-07; T-211-cm-93; T-249-em-85). VPR was Soxhlet extracted with water and ethanol as described in Romaní et al. (2016). Liquors from quantitative acid hydrolysis and aqueous extract were analyzed by High-Performance Liquid Chromatography (HPLC) for sugars (glucose, xylose, arabinose) and acetic acid, using a Refractive Index detector and an 87H (300 × 7.8 mm) Aminex (BioRad) column eluted with 0.005 M H<sub>2</sub>SO<sub>4</sub>, flow rate of 0.6 mL/min at 60 °C. The content of polysaccharides (glucan, xylan, arabinan) and acetyl groups was calculated from HPLC data. The Klason lignin content of VPR was gravimetrically measured from the insoluble solid residue obtained after the quantitative acid hydrolysis step.

## 2.2. Autohydrolysis of vine pruning residue in two sequential stages: solid and liquid characterization

### 2.2.1. First autohydrolysis stage

$$S_0 = \log R_0 = \log(R_0 \text{HEATING} + R_0 \text{COOLING})$$

$$= \log \left[ \int_0^{t_{MAX}} \exp\left(\frac{T(t) - T_{REF}}{\omega}\right) dt \right] + \left[ \int_{t_F}^{t_{MAX}} \exp\left(\frac{T(t) - T_{REF}}{\omega}\right) dt \right] \quad (1)$$

The VPR was submitted to autohydrolysis treatments under conditions listed in Table 1. For this, water was mixed with VPR at liquid to solid ratio (LSR) of 8 g per g, placed in stainless steel reactor and submerged in silicone oil bath in a 160 mL total volume batch cylinder reactor fabricated from 316 stainless steel at distinct temperatures (180–200 °C) and different reaction times (10–90 min) (heating time of 5 min). After autohydrolysis treatment, the reactor was removed from the oil bath and cooled down in an ice-water bath for 5 min. The hardness of autohydrolysis treatments can be expressed in terms of “severity” ( $S_0$ ) (Lavoie et al., 2010), defined as the logarithm of the reaction ordinate ( $R_0$ ) (Overend and Chornet, 1987; Abatzoglou et al., 1992), which was calculated using the following equation:

According to this expression,  $R_0$  is the reaction ordinate (min),  $t_{MAX}$  (min) is the time needed to achieve the target temperature  $T_{MAX}$  (K),  $t_F$  (min) is the time needed for the whole heating–cooling period,  $T(t)$  and  $T'(t)$  represent the temperature profiles in the heating and cooling stages (K), respectively,  $T_{REF}$  is the reference temperature ( $T_{REF} = 373.15$  K) and  $\omega$  is an empirical parameter related with the activation energy of hemicellulose solubilization reaction, which can be expressed as (Garrote et al., 2002):

$$\omega = \frac{RT_{REF}^2}{E_a} \quad (2)$$

where,  $R$  is the ideal gas constant ( $R = 8.314$  J/mol K) and  $E_a$  is the activation energy of hemicellulose solubilization reaction in J/mol. Although  $\omega$  can be evaluated and optimized (Garrote et al., 2002), it is common to select a value of  $\omega = 14.75$  K (González-Muñoz et al., 2011).

After autohydrolysis treatment, solid and liquid phases were separated by filtration for determination of chemical composition. An aliquot of autohydrolysis liquors (liquid phase) was filtered through 0.45  $\mu\text{m}$  membranes and used for direct HPLC determination of glucose, xylose, arabinose, acetic acid, hydroxymethylfurfural (HMF) and furfural, using the same method specified above. A second aliquot was subjected to quantitative post hydrolysis with 4% (w/w) sulphuric acid at 121 °C for 30 min, filtered through 0.45  $\mu\text{m}$  membranes, and analyzed by HPLC for oligosaccharides concentration.

Solid phase from autohydrolysis was washed with distilled water, air-dried and employed for solid yield (SY) determination (expressed as g autohydrolyzed VPR per 100 g VPR, oven dry basis). Pretreated VPR was analyzed for glucan, xylan and Klason lignin using the analytical procedure described in section 2.1.

### 2.2.2. Second autohydrolysis stage

Second autohydrolysis treatment was performed mixing water and pretreated VPR from first autohydrolysis (Fig. 1) at LSR of 6 g per g. Conditions of the second stage of autohydrolysis were listed

**Table 1** Operational conditions of first autohydrolysis treatment at Liquid to Solid Ratio = 8 g per g and main results obtained: composition of solid and liquid phases.

	180	180	180	180	180	180	180	180	180	180	180	200	200	200	200	200
Temperature ( $T_{MAX}$ , °C)	180	180	180	180	180	180	180	180	180	180	180	200	200	200	200	200
Time (min)	10	20	30	40	50	60	70	80	80	80	80	30	40	60	90	200
$S_0$ (-)	3.36	3.66	3.83	3.96	4.05	4.13	4.20	4.26	4.26	4.26	4.26	4.42	4.55	4.72	4.90	62.4
Solid Yield (g per 100 g of raw material)	86.4	83.7	74.8	68.7	68.6	65.7	64.5	63.5	63.5	63.5	63.4	63.4	60.1	60.9	60.9	62.4
<b>Solid Phase Composition (g of component per 100 g of autohydrolyzed VPR oven-dry basis)</b>																
Glucan	36.3 ± 0.45	37.3 ± 0.72	39.3 ± 0.65	38.3 ± 1.82	40.3 ± 1.58	42.6 ± 2.30	45.6 ± 3.74	44.5 ± 2.71	44.5 ± 2.71	44.5 ± 2.71	43.5 ± 1.60	43.6 ± 2.10	43.0 ± 1.96	43.0 ± 1.96	40.7 ± 0.68	40.7 ± 0.68
Xylan	14.07 ± 0.21	17.48 ± 0.01	11.1 ± 0.45	9.14 ± 1.09	8.90 ± 0.23	7.38 ± 0.01	5.48 ± 0.23	5.53 ± 0.03	5.53 ± 0.03	5.53 ± 0.03	3.96 ± 0.43	2.95 ± 0.0	2.37 ± 0.01	2.37 ± 0.01	1.53 ± 0.12	1.53 ± 0.12
Klason lignin	30.6 ± 0.54	31.4 ± 0.03	36.6 ± 1.35	36.6 ± 2.54	40.6 ± 0.32	42.5 ± 1.40	43.8 ± 0.93	42.9 ± 1.80	42.9 ± 1.80	42.9 ± 1.80	44.1 ± 0.80	45.7 ± 0.14	45.2 ± 1.59	45.2 ± 1.59	44.6 ± 0.46	44.6 ± 0.46
Acetyl groups	3.40 ± 0.50	3.18 ± 0.02	1.76 ± 0.58	1.68 ± 0.38	1.52 ± 2.06	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
<b>Liquid Phase Composition (g/L)</b>																
Glucose	0.9 ± 0.01	1.9 ± 0.56	1.9 ± 0.58	1.6 ± 0.39	1.6 ± 0.26	1.3 ± 0.02	1.3 ± 0.15	1.2 ± 0.08	1.2 ± 0.08	1.2 ± 0.08	0.4 ± 0.17	0.7 ± 0.02	0.5 ± 0.04	0.5 ± 0.04	0.2 ± 0.23	0.2 ± 0.23
Xylose	2.5 ± 0.53	2.1 ± 0.16	2.0 ± 0.18	1.8 ± 1.71	1.8 ± 0.13	2.0 ± 0.02	1.8 ± 0.08	2.0 ± 0.21	2.0 ± 0.21	2.0 ± 0.21	2.3 ± 0.37	2.8 ± 0.29	2.0 ± 0.06	2.0 ± 0.06	1.1 ± 0.10	1.1 ± 0.10
Arabinose	2.6 ± 0.24	0.3 ± 0.14	0.7 ± 0.34	0.6 ± 0.81	0.6 ± 0.10	0.4 ± 0.02	0.5 ± 0.13	0.4 ± 0.18	0.4 ± 0.18	0.4 ± 0.18	0.0 ± 0.32	0.0 ± 0.19	0.0 ± 0.11	0.0 ± 0.11	0.0 ± 0.11	0.0 ± 0.11
Acetic Acid	0.16 ± 0.05	0.31 ± 0.14	0.83 ± 0.31	1.13 ± 0.02	1.9 ± 0.02	2.13 ± 0.02	2.66 ± 0.08	2.33 ± 0.23	2.33 ± 0.23	2.33 ± 0.23	3.02 ± 0.36	3.28 ± 0.46	3.51 ± 0.52	3.51 ± 0.52	3.86 ± 0.70	3.86 ± 0.70
HMF	0.1 ± 0.30	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.96	0.1 ± 0.17	0.1 ± 0.02	0.1 ± 0.79	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.70	0.4 ± 0.44	1.0 ± 0.17	1.0 ± 0.17	1.0 ± 0.81	1.0 ± 0.81
Furfural	0.0 ± 0.43	0.1 ± 0.03	0.2 ± 0.05	0.3 ± 0.66	0.3 ± 0.90	0.4 ± 0.02	0.3 ± 0.98	0.4 ± 0.95	0.4 ± 0.95	0.4 ± 0.95	1.2 ± 0.05	2.0 ± 0.64	3.0 ± 0.44	3.0 ± 0.44	2.1 ± 0.27	2.1 ± 0.27
Glucoligosaccharides	5.9 ± 0.52	5.5 ± 4.09	5.0 ± 1.20	4.8 ± 0.28	5.4 ± 0.84	5.0 ± 0.42	5.0 ± 1.01	4.5 ± 0.13	4.5 ± 0.13	4.5 ± 0.13	3.2 ± 0.31	2.5 ± 0.14	1.0 ± 0.04	1.0 ± 0.04	0.3 ± 0.04	0.3 ± 0.04
Xylooligosaccharides	3.4 ± 0.12	3.5 ± 2.97	10.3 ± 1.56	11.3 ± 0.24	12.6 ± 0.59	13.2 ± 0.42	13.0 ± 0.35	13.0 ± 0.72	13.0 ± 0.72	13.0 ± 0.72	12.0 ± 1.01	7.0 ± 0.35	1.7 ± 0.01	1.7 ± 0.01	0.9 ± 0.03	0.9 ± 0.03
Arabinooligosaccharides	2.0 ± 0.18	2.1 ± 0.45	1.4 ± 0.02	0.6 ± 0.57	0.9 ± 0.05	1.1 ± 0.29	0.5 ± 0.19	0.7 ± 0.20	0.7 ± 0.20	0.7 ± 0.20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	0.56 ± 0.39	1.18 ± 3.50	2.73 ± 0.65	2.4 ± 2.57	2.13 ± 0.37	1.97 ± 0.51	1.69 ± 0.11	1.87 ± 0.15	1.87 ± 0.15	1.87 ± 0.15	2.61 ± 0.08	2.7 ± 0.56	2.7 ± 0.31	2.7 ± 0.31	2.08 ± 0.41	2.08 ± 0.41
Total phenolic compounds	1.04 ± 0.08	0.98 ± 0.02	1.01 ± 1.61	1.33 ± 0.03	1.47 ± 0.91	2.09 ± 0.64	1.70 ± 0.65	1.66 ± 0.77	1.66 ± 0.77	1.66 ± 0.77	1.64 ± 0.93	1.91 ± 0.91	1.86 ± 0.31	1.86 ± 0.31	1.99 ± 0.44	1.99 ± 0.44

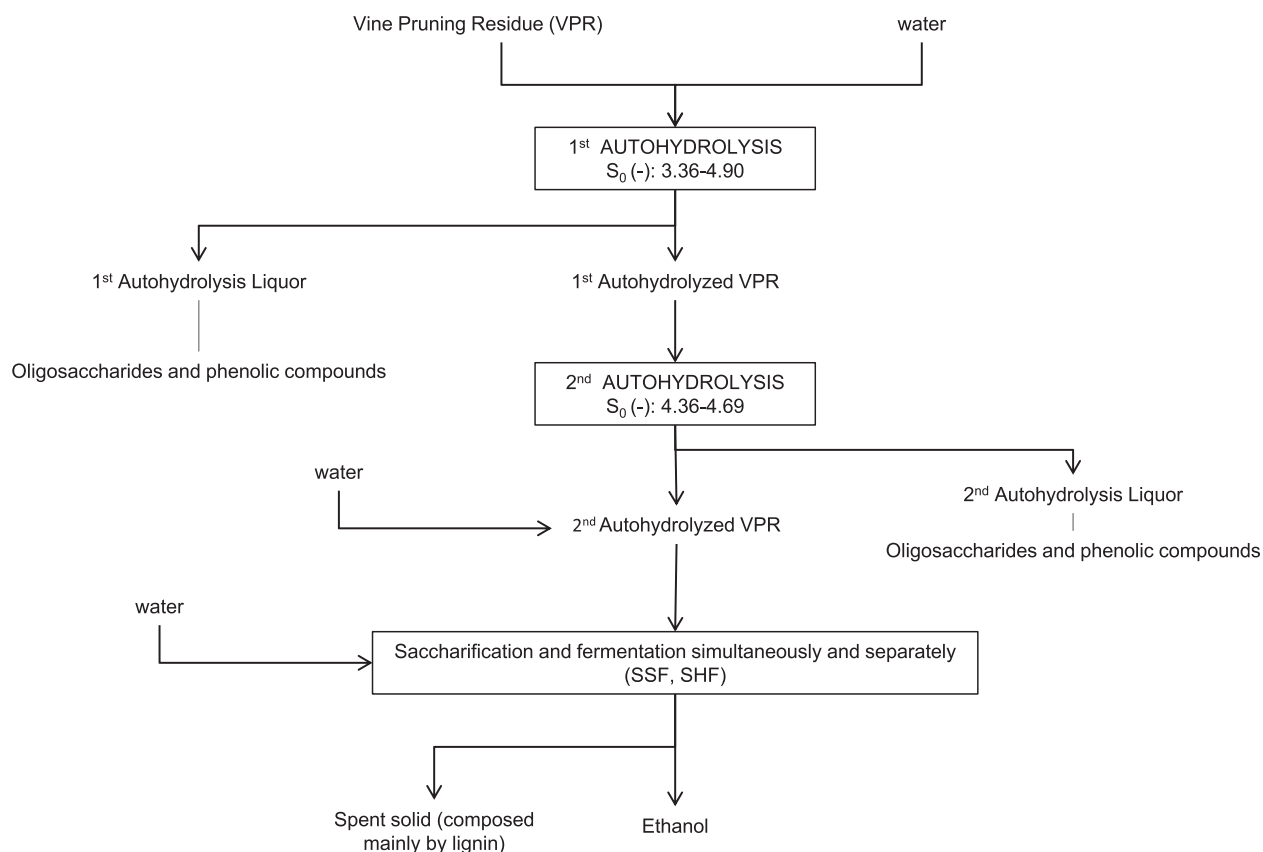


Fig. 1. Scheme proposed in this work for integral valorization of vine pruning residue.

in Table 2 and carried out at LSR of 6 g per g.

### 2.3. Phenolic compounds analysis and antioxidant activity of autohydrolysis liquors

Autohydrolysis liquors were analyzed for total phenolic compounds (expressed as gallic acid equivalents, GAE) by absorbance following Folin-Ciocalteu method (as described in Conde et al., 2011). In addition, some phenolic compounds were also identified by Ultra-high-performance liquid chromatography (UHPLC) using a Shimadzu Nexpera X2 UHPLC chromatograph equipped with Diode Array Detector (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Acquity UHPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; from Waters) at 40 °C. The flow rate was 0.4 mL/min. The HPLC grade solvents used were water/formic acid (0.1%) as solvent A and acetonitrile as solvent B. The elution gradient for solvent B was as follows: from 0.0 to 5.5 min at 5%, from 5.5 to 17 min a linear increase to 60%, from 17.0 to 18.5 min a linear increase to 100%, then column equilibration from 18.5 to 30.0 min at 5%.

Antioxidant activity of autohydrolysis liquors was also determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay and the radical cation decolorization of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay following the methods described in Karacabey et al. (2012) and Ballesteros et al. (2015). The percentage of inhibition was calculated as a function of the concentration of autohydrolysis liquor and Trolox.  $IC_{50}$  (concentration of sample required to reduce 50% of DPPH or ABTS) was calculated by interpolation of the inhibition activity ( $I_a$ , %), calculated by the following equation:

$$I_a = \frac{A_0 - A_1}{A_1} 100 \quad (3)$$

where  $A_0$  is the absorbance of the control (DPPH or ABTS), and the  $A_1$  is the absorbance of the autohydrolysis liquor.

### 2.4. Enzymatic hydrolysis of vine pruning residue from autohydrolysis treatment

In order to evaluate the enzymatic susceptibility of leftover glucan in pretreated vine pruning residue, enzymatic hydrolysis assays were carried out using commercial enzymes Cellic Ctec2 (cellulase) and Htec2 (hemicellulase), kindly provided by Novozymes (Denmark). Enzymatic activities for cellulases and hemicellulases were determined (Ghose, 1987; Bailey et al., 1992) corresponding to 120 Filter Paper Unit (FPU)/mL and 1690 International Unit (IU)/mL, respectively. The enzymatic hydrolysis was carried out in an orbital shaker at 50 °C and 150 rpm using 4% and 10% of solids and enzyme loading of 25 FPU per g of substrate in a 0.05 N sodium citrate buffer (pH 4.85). Samples were withdrawn between 0–72 h and analyzed by HPLC for glucose and xylose concentration. All determinations were performed in duplicate. The results obtained from enzymatic hydrolysis can also be expressed as glucan to glucose conversion (GGC, %) using the following equation:

$$GGC = \frac{G + 1.053 C}{1.111f B} 100 \quad (4)$$

where,  $G$  is glucose concentration (g/L),  $C$  is cellobiose

**Table 2**  
Conditions of second autohydrolysis treatment of VPR from first autohydrolysis ( $S_0 = 4.13$ ) at Liquid to Solid Ratio = 6 g per g and chemical characterization of solid and liquid phases (first and second autohydrolysis stages).

	1st Stage Autohydrolysis		2nd Stage Autohydrolysis			
Temperature ( $T_{MAX}$ , °C)	180		180	180	200	200
time (min)	60		40	60	30	40
$S_0$ (-)	4.13		3.96	4.13	4.42	4.55
Total $S_0^a$ (-)	—		4.36	4.43	4.60	4.69
Solid Yield	66.02		66.60	62.01	62.61	63.60
<i>Solid phase composition</i>	(g per 100 g of 1st autohydrolyzed VPR)		(g per 100 g of 2nd autohydrolyzed VPR)			
Glucan	41.31 ± 3.58		40.40 ± 0.46	41.35 ± 1.70	41.72 ± 0.98	40.35 ± 0.59
Xylan	9.96 ± 2.44		6.98 ± 0.16	6.58 ± 0.20	6.29 ± 0.12	4.28 ± 0.08
Klason Lignin	42.20 ± 0.69		40.05 ± 3.83	40.11 ± 0.53	40.55 ± 2.45	39.58 ± 4.17
<i>Liquid phase composition</i>	(g/L)		(g/L)			
Glucose	1.50 ± 0.79		0.11 ± 0.02	0.14 ± 0.15	0.14 ± 0.03	0.17 ± 0.04
Xylose	1.99 ± 0.93		0.53 ± 0.01	0.81 ± 0.34	1.18 ± 0.03	1.47 ± 0.60
Arabinose	0.72 ± 0.21		0.11 ± 0.01	0.10 ± 0.62	0.12 ± 0.02	0.09 ± 0.09
Acetic Acid	0.96 ± 1.06		0.59 ± 0.09	0.84 ± 0.77	0.98 ± 0.04	1.45 ± 0.24
Furfural	0.36 ± 0.27		0.42 ± 0.02	1.07 ± 0.67	0.99 ± 0.54	1.24 ± 0.52
HMF	0.66 ± 0.42		0.82 ± 0.01	2.47 ± 1.64	3.08 ± 0.88	4.50 ± 0.12
Glucoligosaccharides	6.29 ± 0.63		0.61 ± 0.01	0.55 ± 0.01	0.68 ± 0.02	0.57 ± 0.04
Xylooligosaccharides	17.22 ± 0.73		5.20 ± 0.01	5.50 ± 0.02	6.88 ± 0.12	4.89 ± 0.09
Arabinooligosaccharides	1.60 ± 0.56		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	6.10 ± 0.30		1.74 ± 0.03	1.64 ± 0.02	2.17 ± 0.09	1.82 ± 0.02

<sup>a</sup> Total  $S_0$  was calculated taking into account the severity of first stage and second.

concentration (g/L),  $B$  is dry biomass concentration (g/L),  $f$  is glucan fraction in dry biomass (g per g), the multiplication factor, 1.053, converts cellobiose to equivalent glucose and 1.111 is the stoichiometric factor that converts glucan to equivalent glucose. In all experiments, cellobiose was not detected.

## 2.5. Inoculum preparation

Yeast used in this work was *Saccharomyces cerevisiae* PE-2 strain (isolated from Brazilian Bioethanol Distillery) (Pereira et al., 2014). Stock culture was maintained on yeast peptone dextrose medium (2% of glucose, 2% of peptone and 1% of yeast extract) agar plates at 4 °C. Yeast was grown in Erlenmeyer flasks containing 20 g/L of glucose, 20 g/L of peptone and 10 g/L of yeast extract for 15 h at 30 °C and 200 rpm. Cells were separated from culture media by centrifugation (10 min at 4 °C and 7500 g) and resuspended in 0.9% NaCl. Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) experiments were inoculated with 5 mg of fresh yeast per mL (final concentration).

## 2.6. Saccharification and fermentation

For ethanol production, separate and simultaneous saccharification and fermentation assays (SHF and SSF) were carried out using as substrate the pretreated VPR obtained from autohydrolysis in two sequential stages (Fig. 1). Enzymes used for saccharification were Cellic Ctec2 and Cellic Htec2. Nutrients (peptone and yeast extract) were sterilized in autoclave separately from pretreated VPR at 121 °C for 15 min. Inoculum and enzymes were added to SSF assays. Experiments were carried out at 35 °C and 150 rpm. For SHF assays, enzymatic hydrolysis (at 50 °C) was carried out for 48 h. After this, temperature was decreased to 30 °C and yeast cells were added. Samples from SHF and SSF were withdrawn at desired times and analyzed by HPLC for ethanol concentration. Ethanol yield ( $Y_{Et}$ ) was calculated by the following equation:

$$Y_{Et} = \frac{EtOH_f - EtOH_0}{0.51 f B 1.111} 100 \quad (5)$$

where,  $EtOH_f$  is the ethanol concentration produced during the fermentation (g/L),  $EtOH_0$  is the ethanol concentration at the beginning of the fermentation (g/L) which was zero,  $B$  is dry biomass concentration at the beginning of the fermentation (g/L),  $f$  is glucan fraction of dry biomass (g per g), 0.51 is conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast. The stoichiometric factor that converts glucan to equivalent glucose is 1.111.

## 2.7. Structural analysis of raw material and pretreated samples

### 2.7.1. Scanning electron microscopy (SEM) analysis

Micrographs of raw material, autohydrolyzed vine pruning residue from the first autohydrolysis (180 °C for 60 min,  $S_0 = 4.13$ ) and autohydrolyzed vine pruning residue from the second autohydrolysis (200 °C for 30 min,  $S_0 = 4.60$ ) were obtained using a desktop scanning electron microscope (Phenom-World BV, Netherlands). The images were obtained using a voltage of 5 kV at 270-fold magnification.

### 2.7.2. Thermal analysis of spent residue recovered after SHF assay

Thermogravimetric analysis (TGA) of spent residue recovered after saccharification and fermentation assay was carried out in Thermogravimetric (TGA 4000 and DSC 6000) Analyzer. The analysis was carried out in the range of 25–600 °C with a linear increase of 10 °C per min.

### 2.7.3. Fourier-transform infrared (FT-IR)

The chemical groups and bonding arrangement of constituents of the spent residue obtained after saccharification and fermentation assay were determined by Fourier transform infrared spectroscopy (FT-IR) using a Jasco infrared spectrometer (FT-IR-4100) equipped with a diamond-composite attenuated total reflectance (ATR) cell. The FT-IR spectrum was obtained operating with a resolution of 4  $cm^{-1}$ , 16 scans, and frequency range of 4000–600  $cm^{-1}$ . The FT-IR bands were identified for comparison with those reported in the literature (Santos et al., 2015; Dávila et al., 2017).

### 3. Results and discussion

#### 3.1. Raw material

Chemical composition of vine pruning residue (expressed in g per 100 g VPR on oven-dry basis  $\pm$  standard deviation based in three replicate determinations) was as follows:  $32.9 \pm 0.66$  of cellulose (as glucan);  $14.87 \pm 0.17$  of xylan;  $0.40 \pm 0.06$  of arabinan;  $3.95 \pm 0.52$  of acetyl groups;  $29.5 \pm 1.21$  of Klason lignin;  $13.7 \pm 1.02$  of extractives in water;  $2.94 \pm 0.89$  of extractives in ethanol and  $3.32 \pm 0.56$  of ashes. Structural components of VPR (cellulose, Klason lignin and hemicellulose) represented 81.6% of the raw material. Extractives in water were analyzed by HPLC for determination of glucose concentration which represented 1.20% of raw material oven-dry basis. VPR was composed mainly of cellulose followed by lignin. Hemicellulose fraction was mainly composed of xylan which represented 77.4% of total identified hemicellulose compounds. Chemical composition was similar to the one reported by other authors for vine shoots (Rivas et al., 2007; Dávila et al., 2016).

#### 3.2. Process configuration

Process configuration of this work was shown in Fig. 1. First, autohydrolysis stage was proposed in order to solubilize hemicellulose fraction into added value compounds such as oligosaccharides, recognized prebiotic functional food (Patel and Prajapati, 2015), and phenolic compounds, valued by their bioactive properties and antioxidant activity (Rivas et al., 2013). Autohydrolyzed VPR from the first stage was assayed for enzymatic saccharification. After this evaluation, second autohydrolysis stage was proposed in order to improve the enzymatic susceptibility of cellulose. Finally, pretreated VPR from sequential stages of autohydrolysis was subjected to saccharification and fermentation for ethanol production. A systematic evaluation of operational conditions on fractionation of VPR was carried out as shown below.

#### 3.3. First stage of autohydrolysis pretreatment of vine pruning residue

##### 3.3.1. Fractionation of vine pruning residue

After the first stage of autocytolysis treatment, pretreated biomass and autohydrolysis liquor were analyzed to evaluate the degree of fractionation achieved. Table 1 showed the operational conditions of autohydrolysis treatment (temperature and time and its corresponding severity,  $S_0$ ) and the results obtained. As observed in Table 1, solid yield (SY) decreased with the increase of severity. Glucan content varied in the range of 36.3–45.6 g of glucan per 100 g of autohydrolyzed VPR. The glucan recovery was 77.2–95.2% with respect to glucan content in raw material at  $S_0 = 4.90$  and 3.36, respectively (boundary conditions evaluated in this work). The lowest glucan recovery at  $S_0 = 4.90$  revealed high cellulose loss, that is an undesirable condition for vine pruning processing from a biorefinery approach. Buratti and coworkers (2015) reported a 69.1% of cellulose recovery from steam exploded residue from vineyard at  $S_0 = 4.24$ . The wide range of severities studied in this work displayed the behavior of the raw material to selective fractionation of autohydrolysis treatment. As observed from data listed in Table 1, xylan solubilization increased with the severity of treatment achieving up to 93% of xylan solubilization at  $S_0 = 4.90$ . Lignin content ranged from 30.60 to 45.66 g of lignin per 100 g of autohydrolyzed VPR which corresponded to 90 and 100% of recovery, respectively. Similar range of severities were evaluated for fractionation of agricultural residues as barley straw (Vargas et al., 2015), rice straw (Moniz et al., 2015) or olive tree pruning (Silva-

Fernandes et al., 2015) in which cellulose recovery of 90% were obtained at values of  $S_0$  in the range of 3.15–4.36.

Table 1 also provides the chemical composition of liquors from first autohydrolysis step. As expected, xylooligosaccharides were the main component at milder conditions of treatment ( $S_0 = 3.83$ –4.26). As severity increases, the hydrolysis of oligosaccharides into monosaccharides is more effective but after a critical value leads to the subsequent dehydration of sugars in furfural and HMF. This behavior is typical of the severity rise effect on autohydrolysis treatment of lignocellulosic materials (Garrote et al., 2002; Ruiz et al., 2013). A 16–50% of xylan solubilization into xylooligosaccharides was obtained at  $S_0$  3.36–3.80, achieving a maximal extraction of xylan as xylooligomers (65.1%) at  $S_0 = 4.13$ . The lowest recovery of xylan as xylooligosaccharides (4.4%) was obtained at the highest severity factor evaluated ( $S_0 = 4.90$ ). The maximal concentration (measured as sum) of xylooligosaccharides and xylose (13.19 and 2.01 g/L, respectively) were also obtained at  $S_0 = 4.13$  which corresponded to 75.9% recovery of xylan in the raw material. Similar concentration of xylooligosaccharides (12.2 g/L) or xylose (14.8 g/L) were obtained by autohydrolysis treatment using trimming vine shoots at  $S_0 = 4.01$  (Dávila et al., 2016) and by sequential stages of autohydrolysis at  $S_0 = 4.08$  and acid hydrolysis with 1%  $H_2SO_4$  (w/w) (Moldes et al., 2007), respectively. The concentration of glucooligosaccharides and acetyl groups linked to oligosaccharides achieved an average value of  $5.24 \text{ g/L} \pm 0.39$  and  $2.05 \text{ g/L} \pm 0.67$ , respectively. Regarding degradation compounds, furfural and HMF concentrations were higher than 1 g/L at  $S_0 = 4.42$  and 4.72, respectively. On the other hand, acetic acid from deacetylation of hemicellulose had a significant increase up to 3.86 g/L at  $S_0 = 4.90$ .

Taking into account that by-products from wine-making are enriched in phenolic compounds with valuable antioxidant properties to be used in food, pharmaceutical and cosmetic industries (Delgado-Torre et al., 2012; Teixeira et al., 2014), total phenolic compounds in autohydrolysis liquor were also quantified and included in Table 1. Phenolic compounds obtained by autohydrolysis achieved an average value of  $1.5 \text{ g/L} \pm 0.39$ , achieving a maximal concentration of 2.09 of GAE g/L (or 1.88 g GAE per 100 g of VPR) at  $S_0 = 4.13$ . Autohydrolysis treatment has been employed for extraction of phenolic compounds, obtaining approximately 2 GAE g per 100 g from lignocellulosic wastes such as corncobs, eucalyptus wood and grape pomace at high temperatures (240 °C) in non-isothermal regime (Conde et al., 2011). Traditionally, extraction methods for phenolic compounds recovery from vine by-products use toxic solvents such as methanol (Delgado-Torre et al., 2012). Water extraction at high temperatures is an environmentally-friendly alternative to traditional extraction methods.

##### 3.3.2. Enzymatic susceptibility of vine pruning residue

For a global evaluation of pretreatment, the enzymatic susceptibility of pretreated biomass was also studied. Effect of autohydrolysis pretreatment on time course of enzymatic saccharification was represented in Fig. 2 (assays were carried out in duplicate with a calculated relative error  $\leq 10\%$ ). At  $S_0 = 4.05$ –4.42, glucan to glucose conversion (GGC) at 72 h of enzymatic hydrolysis achieved values of 73–78%. Under these conditions of pretreatment, more than 60% of xylan was solubilized into xylooligosaccharides, disclosing a suitable range of conditions to attain a biorefinery scheme. The highest glucan conversion (91%) was achieved at  $S_0 = 4.90$  in which oligosaccharides and sugars were completely degraded in the liquid phase. Glucose yield higher than 90% was also obtained from alkaline pretreated residue from vineyard using 2.5% NaOH at 100 °C for 30 min (Cotana et al., 2015). Enzymatic saccharification yield of steam exploded residue from vineyard

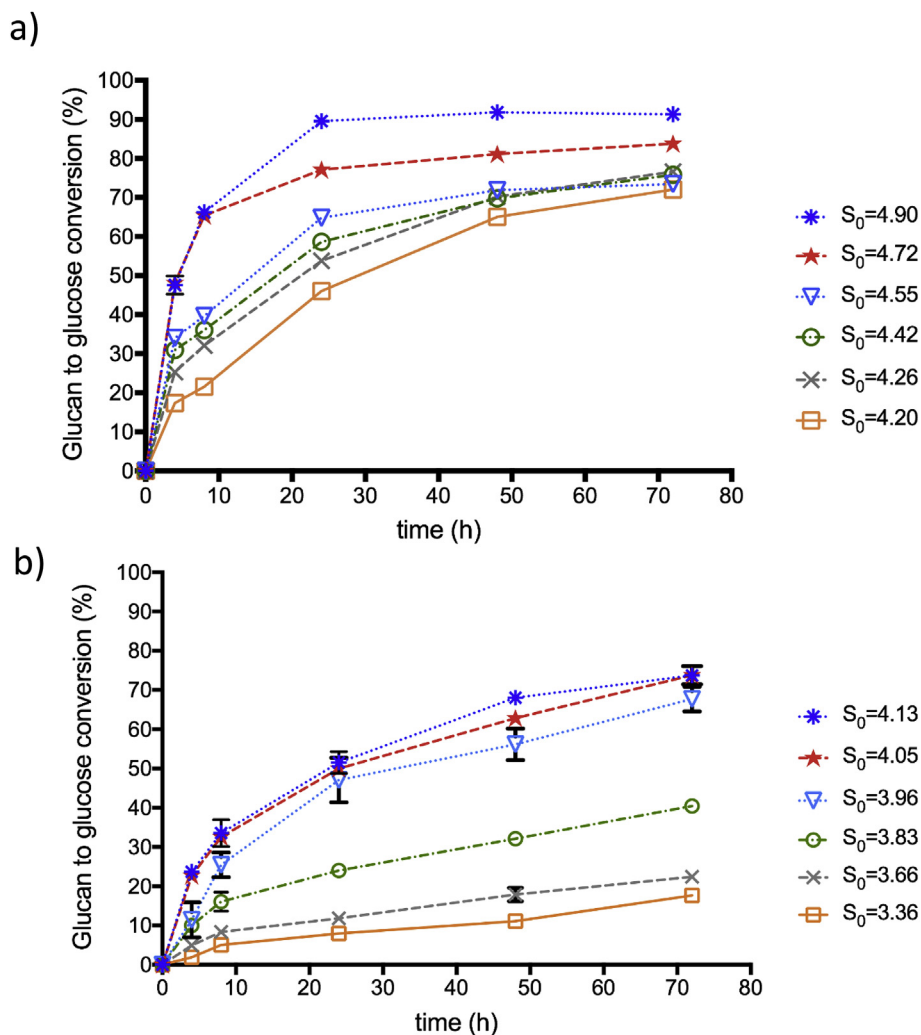


Fig. 2. Time course of cellulose to glucose conversion at  $S_0$  in the range of 3.36–4.90.

attained 86% at  $S_0 = 4.56$  (Buratti et al., 2015). Xylose was also generated in enzymatic hydrolysis assays at a concentration of 0.4–2.3 g/L, corresponding to a xylan conversion of 12–100%, respectively. In this work, the increment in the severity factor significantly enhanced the enzymatic susceptibility of cellulose with 5.4-fold higher conversion comparing to the lowest severity ( $S_0 = 3.36$ ).

Considering the autohydrolysis liquor composition in xylooligosaccharides (13.19 g/L) and enzymatic susceptibility of solid phase (glucan to glucose conversion of 73.7%), operational condition of  $S_0 = 4.13$  was selected to advance on further fractionation of VPR. For that, an additional treatment was carried out reducing the LSR up to 6 g per g with the objective of reducing water consumption in the treatment and increase the concentration of hemicellulose-derived compounds in autohydrolysis liquor. The solid and liquid phase composition was shown in Table 2. As seen, oligosaccharides concentration was considerably increased up to 31.2 g/L (17.2 g/L as xylooligosaccharides).

Some of the phenolic compounds present in the autohydrolysis liquor were identified by UHPLC. Phenolic acids from wine by-product include benzoic and cinnamic acid derivatives, being hydroxycinnamic acid the most abundant in these residues (Teixeira et al., 2014). In accordance, hydroxycinnamic acids were the ones found in the highest percentage in the autohydrolysis

liquor (caffeic acid, p-coumaric acid, chlorogenic acid, rosmarinic acid and ferulic acid) (Table 3). Moreover, hydroxybenzoic acids such as vanillic, gallic and syringic acid were also identified. Grape stems constitute an enriched source of flavonoids and stilbenes (containing resveratrol in high concentration) (Anastasiadi et al., 2012). More recently, shoots, leaves and tendrils from six grapevine varieties were analyzed for their content in resveratrol in which 9.25–12.5 mg of trans-resveratrol per kg of shoots from *St. Laurent vine* variety was reported using an extraction method with methanol: ethyl acetate (Lachman et al., 2016). In this study, the extraction of resveratrol from VPR by autohydrolysis achieved a concentration of 12.46 mg/L (or 79 mg per kg of VPR).

Antioxidant activity of autohydrolysis liquor was also reported in Table 3 showing antioxidant capacity expressed as Trolox equivalent. Antioxidant activity of phenolic compounds obtained by autohydrolysis treatments, liquid hot water and steam explosion, of olive pruning was reported to be 7.33 and 6.31 mmol DPPH per 100 g of extract, respectively (Conde et al., 2009). Recently, antioxidant, antifeedant and phytotoxic activities of aqueous extracts from vine-shoots have been reported showing the potential of this residue to be applied in cosmetics, nutraceuticals or pharmaceuticals (Sánchez-Gómez et al., 2017). Values  $IC_{50}$  for extract samples from vine shoots of 32  $\mu\text{g/mL}$  and 38  $\mu\text{g/mL}$  were reported using conventional solid-liquid extraction and microwave

**Table 3**  
Antioxidant activity and phenolic compounds composition in liquors from first stage ( $S_0 = 4.13$ ) and second stage ( $S_0 = 4.60$ ) of autohydrolysis.

Severity Factor ( $S_0$ )	4.13	4.60
<i>Antioxidant activity</i>		
ABTS (mg per L Trolox equivalent)	247.05 ± 0.34	35.56 ± 0.03
DPPH (mg per L Trolox equivalent)	173.45 ± 0.03	45.2 ± 0.21
ABTS IC <sub>50</sub> (mg of autohydrolysis liquor per mL)	10.97 ± 0.32	50.2 ± 0.15
DPPH IC <sub>50</sub> (mg of autohydrolysis liquor per mL)	9.89 ± 0.58	57.25 ± 0.25
Total phenol (GAE g/L)	2.35 ± 0.02	1.43 ± 0.06
Phenolic Compound	(mg/L)	(mg/L)
catechin	47.54	14.86
syringic acid	18.88	12.65
chlorogenic acid	34.70	0.0
caffeic acid	33.60	0.0
vanillic acid	21.96	16.57
ferulic acid	5.0	0.0
gallic acid	8.39	5.06
p-coumaric acid epicatechin	5.54	4.60
o-coumaric acid	10.32	4.72
hesperidin	21.84	0.0
cinnamic acid	7.26	6.91
resveratrol	12.46	11.78
rutin	20.62	10.40
rosmarinic acid	12.24	11.72

extraction, respectively (Sánchez-Gómez et al., 2017).

### 3.4. Second stage of autohydrolysis pretreatment of vine pruning residue

#### 3.4.1. Effect on solid and liquid composition

Sequential second stage of autohydrolysis was studied for the improvement of enzymatic cellulose susceptibility. VPR from autohydrolysis treatment at  $S_0 = 4.13$  and LSR of 6 g per g was employed as feedstock in a second autohydrolysis step in the range of temperatures from 180 to 200 °C for 30–60 min, corresponding to total  $S_0 = 4.36$ –4.69 (including the severity from the first stage of autohydrolysis). Table 2 collected solid and liquid composition from autohydrolysis in two sequential stages. Under the evaluated conditions of treatment, high glucan recovery was obtained (94.0–96.6 g of glucan per 100 g of glucan in first autohydrolyzed VPR). Glucan content increased with the severity factor achieving 41.7 g of glucan per 100 g of second autohydrolyzed VPR at  $S_0 = 4.60$ . These results were comparable with glucan obtained from first autohydrolysis stage for  $S_0$  of 4.55 and 4.72 (Table 1). Xylan recovery was in the range of 67.5–41.4 g of xylan per 100 of xylan in first autohydrolyzed VPR for the boundary conditions ( $S_0$ : 4.36 and 4.69, respectively). On the other hand, lignin recovery remained almost constant with an average value of  $90.56 \pm 0.96$  g of lignin per 100 g of lignin in the first autohydrolyzed solid. Consequently, the main compound in the liquid phase (or liquor from second autohydrolysis) were xylooligosaccharides which achieved a maximum concentration (6.88 g/L) at  $S_0 = 4.60$ . Considering the xylooligosaccharides extraction in the first autohydrolysis stage, 81.2% of xylan was recovered as xylooligosaccharides from first and second autohydrolysis stages (73.4 and 18.4% at  $S_0 = 4.13$  and  $S_0 = 4.60$ , respectively). Moreover, phenolic compounds present in the liquor from second autohydrolysis were also analyzed by UHPLC and included in Table 3. The concentration of identified phenolic compounds in the second step of autohydrolysis was reduced. Nevertheless, the antioxidant activity was similar probably caused by the higher concentration of HMF (3.8 g/L) formed due to the harder conditions of the second autohydrolysis.

#### 3.4.2. Effect on enzymatic susceptibility of vine pruning residue

Regarding enzymatic hydrolysis from the second stage of autohydrolysis, Table 4 showed operational conditions evaluated and the main results obtained at 72 h of hydrolysis. The percentage of solids was increased from 4% up to 10% in order to increase glucose concentration and consequently ethanol in subsequent fermentation assays. Glucan to glucose conversion was significantly increased, achieving 99% of conversion at the highest severity ( $S_0 = 4.69$ ). The maximal concentration of glucose (45.4 g/L) was obtained at  $S_0 = 4.69$  and 10% of solids. The increase of solid loading up to 10% reduced the glucan to glucose conversion between 1.6–4.6% achieving a glucose concentration 2.2-fold higher than the concentration obtained when operating at 4% of solids. Glucan conversion from  $S_0 = 4.60$  was higher than 80%. In comparison with the saccharification of first autohydrolysis stage ( $S_0 = 4.13$ ), an increase of 35.6% of enzymatic hydrolysis conversion was obtained at  $S_0 = 4.69$  and 4% of solids. On the other hand, 43.7% augment in glucose conversion was achieved using 10% of solid loading and severity of  $S_0 = 4.69$ .

The improvement of enzymatic hydrolysis after autohydrolysis in two sequential stages can be due to structural changes. Scanning Electronic Micrographs (SEM) of raw material and pretreated samples were taken (see Fig. 3). These treated samples correspond to treatment at 180 °C for 60 min (first autohydrolysis,  $S_0 = 4.13$ ) and 200 °C for 30 min (second autohydrolysis, Total  $S_0 = 4.60$ ). Clear differences between native and pretreated biomasses were shown. The raw material displayed an ordered structure of fibers. On the other hand, pretreated sample from the first autohydrolysis showed a more porous structure and the sample from autohydrolysis in two sequential stages presented a more defragmented and open structure.

Few works have reported the effect of autohydrolysis in two stages on lignocellulosic biomass being the results obtained in this work favorably compared with the literature (Park et al., 2016; Williams et al., 2016). Recently, this strategy has been employed to reduce the inhibitor loading in wheat straw and hardwoods hydrolysates (Min et al., 2015; Park et al., 2016). It was also employed for the improvement of enzymatic cellulose saccharification, an increase from 67 to 75% of glucose yield with 30 FPU per g was obtained by two steps of autohydrolysis using coastal Bermuda grass (Lee et al., 2010). On the other hand, combined autohydrolysis treatment at  $S_0 = 4.66$  achieved a 66% of sugar recovery from wheat straw (Min et al., 2015).

#### 3.5. Bioethanol production by separate and simultaneous saccharification and fermentation

Taking into account the results discussed in the above section, VPR treated by autohydrolysis in two sequential stages (Total  $S_0 = 4.60$ ) was chosen for ethanol production. At this condition, 96% of glucan was recovered after treatment, xylooligosaccharides in autohydrolysis liquor achieved the highest concentration (6.9 g/L) and enzymatic saccharification conversion was higher than 80%. For ethanol production, two strategies were evaluated: separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Operational conditions for SHF and SSF were included in Table 5. SHF experiments were shown in Fig. 4a (assays were carried out in duplicate with a calculated relative error  $\leq 10\%$ ). Glucose concentration achieved the maximal concentration of 47.2 and 16.5 g/L for 10 and 4% of solids, respectively. After 48 h of enzymatic hydrolysis, yeast cells were added achieving maximal ethanol concentration within 4 and 8 h of fermentation with an ethanol yield > 90%.

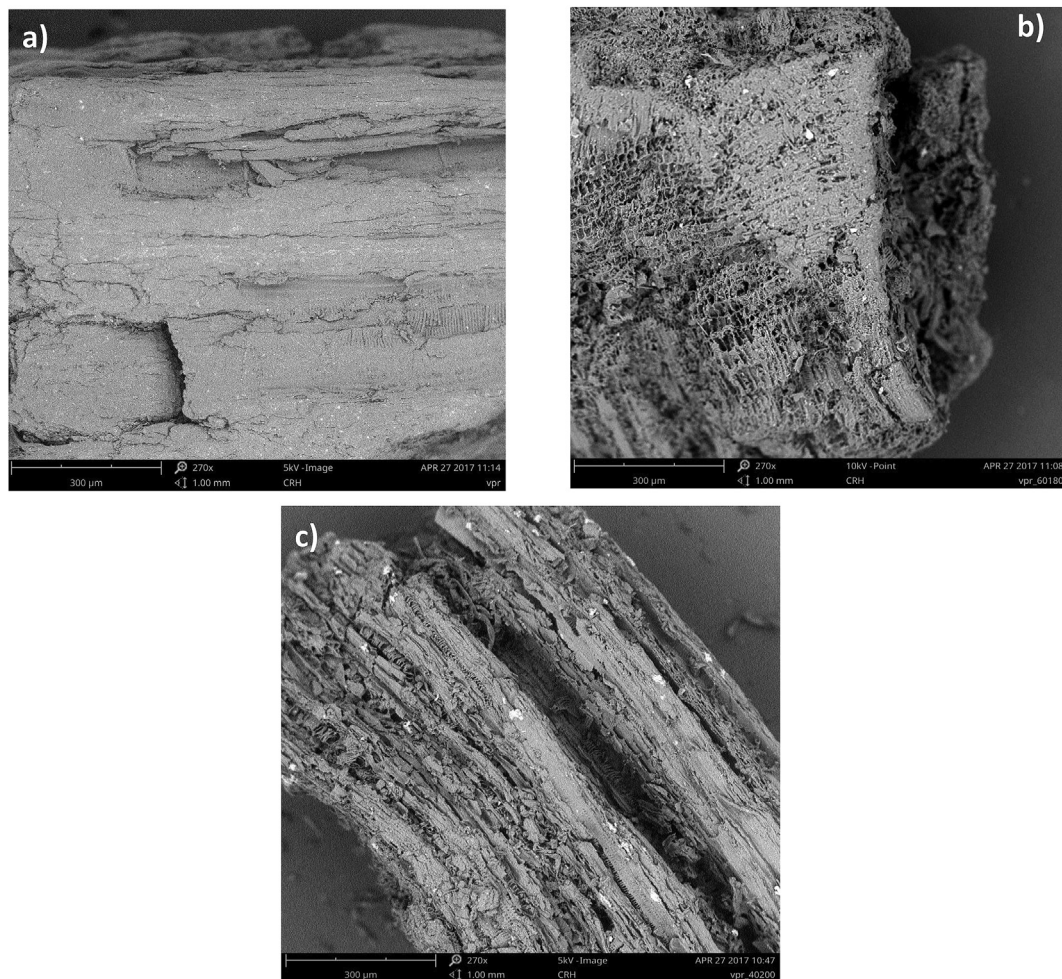
SSF has been proposed as a strategy for improvement of ethanol yield since glucose is simultaneously produced and consumed



**Table 4**  
Enzymatic hydrolysis conditions and main results obtained from saccharification of second autohydrolysis stage at 72 h.

run	Operational conditions				Main Results					
	Temperature (°C)	Time (min)	Severity ( $S_0$ ) or Total Severity (Total $S_0^a$ )	Solid loading (%)	Enzyme to Substrate Ratio (ESR, FPU per g)	Glucose at 72 h (g/L)	Xylose at 72 h (g/L)	Glucan to glucose Conversion (%)	Xylan to glucose Conversion (%)	
1	180	60	4.13	4	25	13.20 ± 0.01	3.51 ± 0.30	73.21 ± 0.06	77.5 ± 0.74	
2				10	25	29.85 ± 0.34	8.21 ± 0.12	68.45 ± 0.78	72.5 ± 2.99	
3	180	40	4.36 <sup>a</sup>	4	25	12.80 ± 0.28	3.05 ± 0.10	72.63 ± 1.60	96.1 ± 2.56	
4				10	25	27.55 ± 0.07	7.80 ± 0.05	70.84 ± 3.18	98.3 ± 1.28	
5	180	60	4.46 <sup>a</sup>	4	25	13.47 ± 0.13	2.88 ± 0.25	74.69 ± 0.75	96.3 ± 6.40	
6				10	25	34.95 ± 2.89	7.20 ± 0.20	71.25 ± 6.65	96.3 ± 0.43	
7	200	30	4.60 <sup>a</sup>	4	25	15.50 ± 0.14	2.80 ± 0.10	85.16 ± 0.91	97.9 ± 2.56	
8				10	25	40.50 ± 1.55	7.05 ± 0.34	83.21 ± 3.53	98.6 ± 0.78	
9	200	40	4.69 <sup>a</sup>	4	25	17.48 ± 0.74	1.91 ± 0.30	99.30 ± 4.21	98.2 ± 7.69	
10				10	25	45.43 ± 0.03	4.80 ± 2.86	97.70 ± 0.08	98.7 ± 5.98	

<sup>a</sup> Total  $S_0$  was calculated taking into account the severity of first stage and second.



**Fig. 3.** Scanning Electron Microscopy images: a) vine pruning residue; b) autohydrolyzed vine pruning residue from first autohydrolysis treatment (180 °C for 60 min,  $S_0$  of 4.13); c) autohydrolyzed vine pruning residue from sequential stages of autohydrolysis (200 °C for 40 min, Total  $S_0$  of 4.60).

avoiding end-product inhibition of cellulose enzymes (López-Linares et al., 2014). In this sense, solid loading was increased up to 16.7% and enzyme loading was decreased (15 FPU per g) to evaluate the SSF strategy under demanding conditions. Ethanol profiles of SSF experiments were shown in Fig. 4b (assays were carried out in duplicate with a calculated relative error  $\leq 10\%$ ). Ethanol production was affected by a reduction of enzyme loading achieving low ethanol yields ( $Y_{Et} = 52.9\%$ ) (Table 5). Ethanol

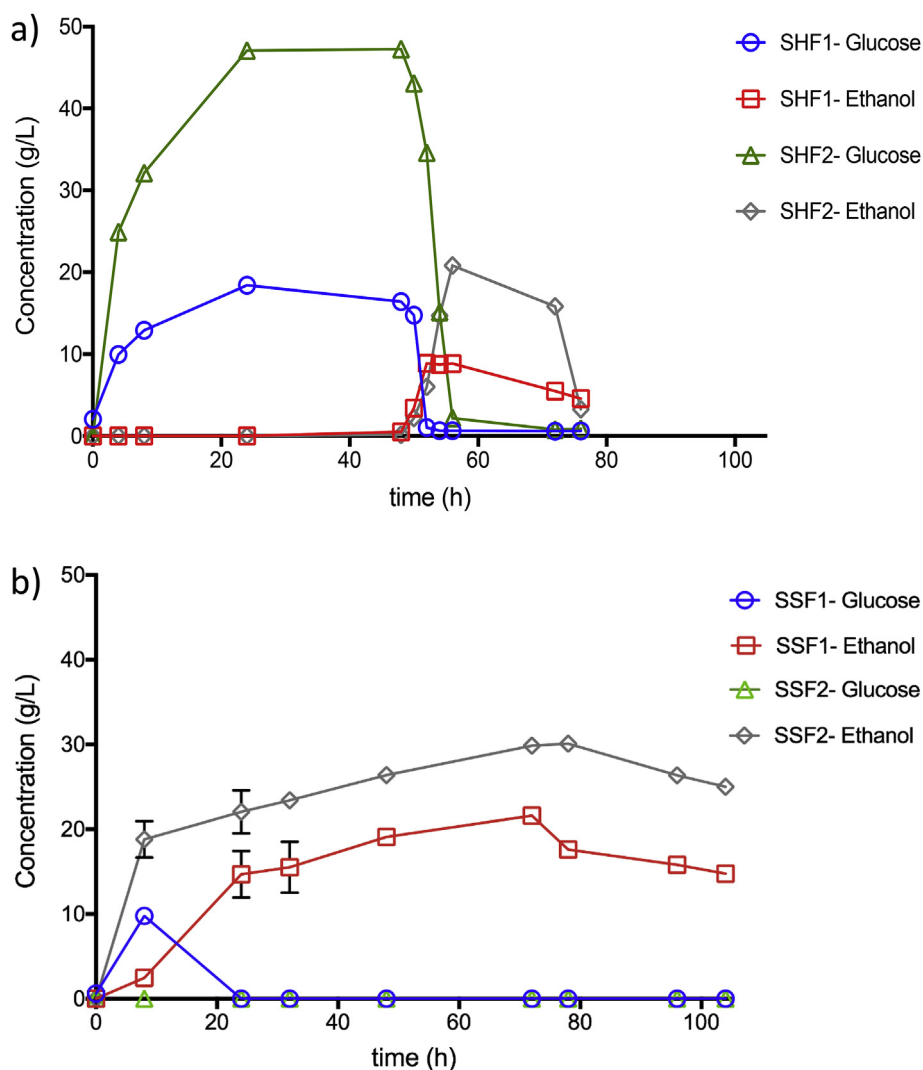
concentration of 30 g/L corresponding to an  $Y_{Et}$  of 83% was obtained with enzyme loading of 25 FPU per g. Ethanol production was strongly affected by the enzyme loading. Nevertheless, recent studies on enzyme recycling suggested the possibility of enzyme recovery being used in sequential batches reducing the overall enzyme loading (Gomes et al., 2016).

Ethanol results obtained in this work can be compared with reported data using residues from wine-making process. SHF

**Table 5**

Operational conditions of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) and main results obtained from vine pruning residue from sequential stages of autohydrolysis (Total  $S_0 = 4.60$ ).

Run	Operational conditions		Main results	
	Solid loading (%)	Enzyme Substrate Ratio (FPU per g)	Ethanol max (g/L)	Ethanol yield, $Y_{Et}$ (%)
SHF1	4	25	$8.84 \pm 0.08$	$95.26 \pm 0.86$
SHF2	10	$20.97 \pm 0.21$	$93.50 \pm 1.07$	
SSF1	16.7	25	$30.08 \pm 0.85$	$83.41 \pm 2.36$
SSF2	16.7	15	$19.09 \pm 0.33$	$52.94 \pm 0.92$



**Fig. 4.** a) Separate hydrolysis and fermentation experiments using 4% (SHF-1) and 10% (SHF-2) of solids and 25 FPU per g and b) Simultaneous saccharification and fermentation (SSF) using 16.7% of solids and 25 (SSF-1) and 25 FPU per g (SSF-2).

strategy was also used for ethanol production from residue derived from vineyard pruning treated by steam explosion ( $S_0 = 4.56$ ), obtaining 8.8 g/L of ethanol with 81.09% of ethanol yield (Buratti et al., 2015). By-products from wine-making processing such as grape skins (containing water-soluble carbohydrates) have been used as raw material for ethanol production obtaining a maximal concentration of ethanol (22 g/L) with ethanol yield of 0.51 g per g using sequential treatments of hexane and water extraction and acid hydrolysis (Mendes et al., 2013). Pretreated grape stalks by autohydrolysis followed by acid hydrolysis were also used for ethanol production obtaining 20.84 g/L (0.35 g ethanol per g

sugars) (Egüés et al., 2013).

### 3.6. Recovered spent residue after saccharification and fermentation process

Finally, spent solid residue was recovered after SHF (using 4% solids) and analyzed for chemical composition. Results showed a solid residue mainly composed by lignin (80.7 g of Klason lignin per 100 g of spent solid from SHF). This result can be compared to Klason lignin from isolated lignins (81–86%) obtained by autohydrolysis followed by organosolv, acetosolv and alkali processes

(Dávila et al., 2017). The chemical structure of the recovered residue was analyzed by FT-IR (Fig. 5a). The FT-IR analysis is used to determine the presence of functional groups, lignin purity and lignin units composition (Santos et al., 2015). The spectrum showed the characteristic bands for lignin functional groups: hydroxyl group and phenol compounds between  $3500$  and  $3000\text{ cm}^{-1}$ , aromatic ring in the region of  $1500\text{ cm}^{-1}$ , carbonyl and carboxyl groups in the band of  $1700\text{ cm}^{-1}$ . The FT-IR spectrum of spent residue from SHF also showed an intensity of monolignols present in the structure of lignin: signal assigned to syringyl units (the bands at  $1330$  and  $833\text{ cm}^{-1}$ ) and guaiacyl units (the bands observed at  $1511$ ,  $1421$  and  $913\text{ cm}^{-1}$ ). This spectrum can be compared with FT-IR spectra from isolated lignins obtained by sequential stages of autohydrolysis and organosolv, acetosolv and  $8\%$  NaOH of vine shoot (Dávila et al., 2017).

On the other hand, thermogravimetric analysis (TGA) of this sample was also carried out (Fig. 5b), weight loss (4.7%) at  $100\text{ }^{\circ}\text{C}$

was due to moisture removal. In the range of  $100\text{--}280\text{ }^{\circ}\text{C}$ , the weight loss was almost constant (corresponding to 6%). The highest weight loss (30%) was achieved at  $368\text{ }^{\circ}\text{C}$ , which was similar to those reported by previous workers using lignin extracted from enzymatic hydrolysis process (Tana et al., 2016). In the range of  $420\text{--}900\text{ }^{\circ}\text{C}$ , the weight loss (20%) was less pronounced. Final residue yield for spent solid recovered after SHF was 30%, corresponding to ashes and protein contents (Tana et al., 2016). Moreover, residual yeast from fermentation process is also present in this kind of residues (Yunus et al., 2015). Therefore, the use of spent residue from saccharification and fermentation process as new protein and antioxidants source could be an interesting alternative leading to widened valorization of VPR.

#### 4. Conclusions

In this work, autohydrolysis in two sequential stages was

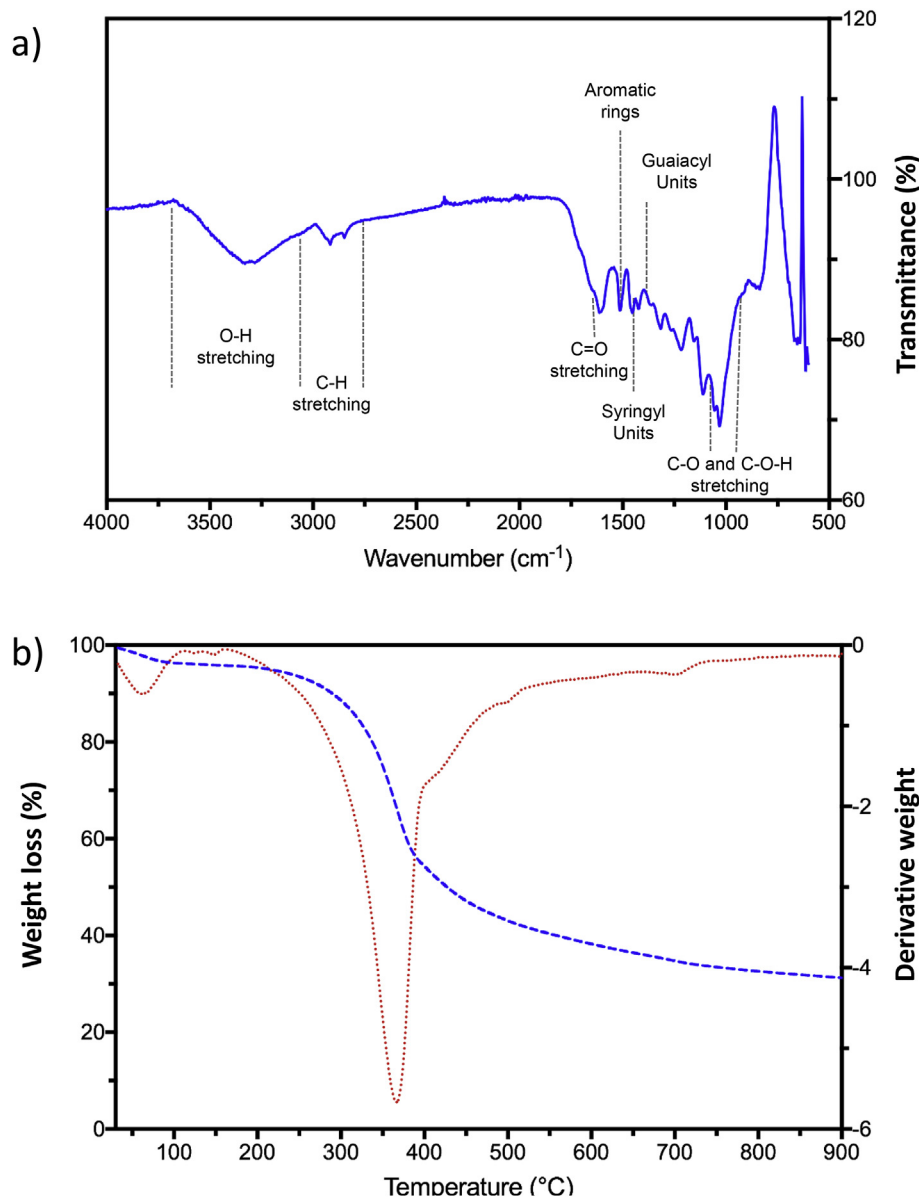


Fig. 5. Analysis of spent residue after enzymatic saccharification and fermentation: a) FT-IR spectrum; b) Thermogravimetric analysis (TGA) and its derivative thermogravimetric (DTG) curve.

proposed for the integral valorization of VPR. High concentration of oligosaccharides with antioxidant activity was obtained from the first step of autohydrolysis. The second autohydrolysis improved the enzymatic saccharification of VPR. Selected conditions led to the following products yield (per 100 kg of VPR): 13.6 kg of xylooligosaccharides, 3.1 kg of phenolic compounds, 13.1 kg of ethanol and 27.0 kg of lignin in four separate streams. As a whole, 69 kg of value added compounds were obtained from processing of 100 kg VPR being suitable for manufacture in energy, pharmaceutical and food industries.

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