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Valorisation of Stalks from Different Grape Cultivars for Sugars Recovery

Giorgia Spigno*, Alessandro Moncalvo, Dante M. De Faveri, Angela Silva

Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza (Italy)
giorgia.spigno@unicatt.it

This study has been realized under a national project Valorvitis focused on the valorisation of wine making by-products for the production of high added-value compounds. The aim of this work was to investigate the possibility of recovering sugars as hemicelluloses hydrolysates suitable for bioethanol production. The production of hemicelluloses hydrolysates from stalks of six different grape cultivars was studied to assess: the cultivar influence on the recovery of non-structural sugars in the washing pre-treatment step; the influence of the cultivar and of the liquid/solid ratio in the acid hydrolysis step on the recovery of structural sugars; and the cultivar influence on the content of sugars degradation products(5-HMF, furfural and 5_MF), total phenols (based on Folin index), phenolic acids (by HPLC) and minerals in the hydrolysates (since these compounds could positively or negatively influence fermentation processes). The applied process consisted in a washing pre-treatment, followed by a dilute acid hydrolysis step (2 % sulfuric acid with two different solvent-to-solid ratios (10:1 and 20:1). The pre-treatment allowed to recover from 4.7 to 6.8 $g_{(Glucose+Fructose)}/100\ g_{dm}$ depending on the cultivar, but in very diluted liquors (< 5 g/L as Glu+Fru). The 10:1 ratio was selected for the hydrolysis, since allowing for almost the same xylose yield as for the 20:1 ratio (from 4 to 7.4 $g/100\ g_{dm}$ depending on the cultivar), the liquors contained lower amounts of phenolic compounds and sugar degradation products. The antioxidant activity (based on the radical ABTS test) of the hydrolysates (which could have an inhibitory effect in fermentation) was slightly influenced by the cultivar but not by the solvent to solid ratio. For all the cultivars, mineral analysis showed a certain content in micro and macronutrients that could positively influence a hypothetical fermentation process for bioethanol production. Therefore, the applied process could be optimized in order to recovery higher quantity of sugars with a reduced generation of inhibitory substances, to be used in fermentation processes.

1. Introduction

Wine industry is one of the most important activities that contribute substantially to national economics in many countries. The main by-products generated by winemaking industry are grape pomace followed by stalks (10–20 % and 2–8 % of processed material, respectively) (González-Centeno et al., 2010). Grape stalks are the skeleton of the grape bunch and consist in lignified tissues with an important concentration of lignin, cellulose and hemicelluloses that, if fractionated, can represent a good renewable organic source. Hemicelluloses, which represent the second most abundant biopolymer in plant kingdom, can be further hydrolysed to give fermentable sugars that, after appropriate purification, could be used in industrial fermentation processes for the production of biochemicals. Many studies have been focused in the hemicelluloses hydrolysates use to obtain biofuels. Previous works have investigated the recovery of cellulose, hemicelluloses, lignin and antioxidants from grape stalks applying different lignocellulosic fractionation processes: acid and alkaline/oxidative hydrolysis (Spigno et al., 2008; Spigno et al., 2013), autohydrolysis and organosolv process (Amendola et al., 2012; Egüés et al., 2013). The results have shown a significant influence of the cultivar on the stalk response to fractionation, a general difficulty in obtaining well delignified cellulose fractions and the possibility of recovering sugars as hemicelluloses hydrolysates suitable for bioethanol production even without any purification step. The presence on the

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stalks of non-structural carbohydrates, which are sugars derived from residual impregnated grape juice, makes necessary a washing pre-treatment before fractionation processes to avoid the production of toxic sugar degradation compounds (SDC) as 5-hydroxymethylfurfural (5-HMF), furfural and 5-methylfurfural (5-MF). Furthermore, the presence of phenolic compounds and metals in hydrolysates should be assessed to verify their positive or negative effect on fermentation process.
In this context, the objectives of this study were: to investigate the possibility of recovering sugars during the washing pre-treatment of stalks from different grape cultivars; to verify the influence of the cultivar on the hemicelluloses hydrolysates production (carried out with dilute sulfuric acid and at two different solvent-to-solid ratios), and to assess the presence of metals, phenolic and sugars degradation compounds in the obtained hydrolysates.

2. Materials and methods

2.1 Washing and hydrolysis process

Grape stalks were obtained during vintage from three red (Barbera, Pinot noir, Nebbiolo) and three white cultivars (Moscato, Chardonnay, Müller Thurgau) in Northern Italy. They were oven-dried at 60°C for 24-48 h and milled (final particle size ≤ 2 mm). The applied process was that reported by Spigno et al. (2013) with slight modifications. Briefly, a washing pre-treatment was carried out (water:stalks ratio 14:1 mL:g; under stirring at 40°C at 180 rpm for 2 h in an orbital shaker, INFORS AG CH-4103 Bottmingen Switzerland), followed by filtration and centrifugation (2,800 g for 15 min) and analysis of the recovered liquid phase for sugars content (glucose and fructose by Megazyme enzymatic kit). The volume of the recovered liquor was measured and the imbibition ratio (r) as mL of water imbibed per g of stalks was calculated. Washed stalks were dried at 50 °C until constant weight and then hydrolysed with 2% H_2SO_4 in autoclave at 121 °C for 90 min with two different solvent-to-solid ratios (SSR) (10:1 and 20:1 mL:g). After hydrolysis, the samples were let cooling partly in the autoclave and partly with cold water, and the hydrolysates were separated by filtration and centrifugation (2,800 g for 15 min) and analysed for the following parameters.

2.2 Analysed parameters

- Reducing sugars concentration was evaluated by Megazyme kits (based on previous works, glucose and xylose were assessed being the main sugars, and the absence of fructose was also verified).
- Colour was measured by absorbance at 440 nm.
- Total phenolic compounds content (TPC) was determined by Folin-Ciocalteau analysis (Folin Index), expressed as gallic acid equivalents (GAE) based on a calibration curve with standard gallic acid (García et al., 2011).
- Total cinnamic acids content was determined by absorbance reading at 320 nm, expressed as ferulic acid equivalents (FE) based on a calibration curve with standard ferulic acid (Spigno et al., 2007);
- Antioxidant activity was evaluated according to the radical ABTS test and expressed as percentage inhibition of radical oxidation (AOP%) (García et al., 2011).
- Phenolic profile was determined by HPLC. The sample pH was adjusted at 3.6 by NaOH 12 N, subsequently phenolic compounds were extracted by columns (SupercleanTM ENVITM-Chrom P SPE Tubes 6 mL 0.50 g) according to the method reported by Mazzoleni et al. (1998). Gallic acid, furfural, 5-HMF and 5-MF were quantified by direct injection after sample filtration (0.20 µL). Analysis was carried out by HPLC (Perkin Elmer - Norwalk, CT, USA – 200 Series pump), with a Supelcosil^{TM} LC-18 (250x4.6; 5 µm) column, and detection by UV-Vis absorption with DAD scanning between 280 and 320 nm. The identification of phenolic compounds and SDC was obtained by authentic standard additions and by comparing the retention times, while quantification was performed by external calibration with standards.
- Mineral content (Ca, Mg, K, Zn, Fe, Cu, Mn) was analysed by atomic absorption spectroscopy (OIV, 2013).

2.3 Statistics

Each trial was carried out in three replicates and the values reported as means ± SD. IBM SPSS® 19.0 (SPSS, Chicago, IL, USA) software for Windows was used to perform statistical analysis of variance (ANOVA) followed by Tukey's post hoc test (for means discrimination) to assess the significance of variation among the six varieties. Variance homogeneity was confirmed according to Levene's test. All significance tests were conducted at $P \leq 0.01$.

3. Results and discussion

3.1 Washing pre-treatment

Table 1 reports the results of the washing pre-treatment (recovery values were calculated based on the total volume of water used for the process). Considering only the recovered liquor (based on the imbibition ratio which was not influenced by the cultivar), this step allowed to recover a total amount of glucose and fructose from 4.7 to 6.8 g / 100 g of stalks dry matter (dm) depending on the cultivar, with a glucose/fructose ratio <1 in all the varieties, apart from Müller Thurgau. However, the washing liquors were very diluted (< 5 g/L as Glu+Fru) making the sugars recovery an expensive process. However, further trials with a lower water:stalks ratio could allow for more concentrated liquors, revealing still an economic interest in the recovery of these sugars, considering that this step is needed in order to reduce the production of SDC in the next hydrolysis step.

Table 1: Influence of cultivar on sugars recovery in the washing pre-treatment. dm: dry matter of stalks. The values followed by different superscript letters in the same column were statistically different.

Cultivar	Imbibition ratio (mL/g)	Glucose g/L	$g_{glucose}$/100 g_{dm}	Fructose g/L	$g_{fructose}$/100 g_{dm}
Chardonnay	3.99±0.30	1.90±0.01^{c}	2.96±0.01^{d}	2.47±0.10^{c}	3.85±0.16^{b}
Moscato	4.06±0.21	1.37±0.05^{b}	2.10±0.08^{b}	2.32±0.01^{bc}	3.54±0.02^{b}
Müller Thurgau	4.00±0.28	1.94±0.11^{c}	2.98±0.18^{d}	1.53±0.07^{a}	2.35±0.11^{a}
Barbera	4.02±0.29	1.56±0.01^{b}	2.44±0.03^{c}	2.22±0.03^{b}	3.49±0.05^{b}
Nebbiolo	3.89±0.14	1.48±0.03^{b}	2.17±0.05^{bc}	1.75±0.10^{a}	2.57±0.14^{a}
Pinot noir	3.94±0.24	1.01±0.04^{a}	1.54±0.07^{a}	2.31±0.01^{bc}	3.52±0.01^{b}

3.2 Acid hydrolysis

The hydrolysate colour is directly related to the presence of lignin degradation products. Therefore, the absorbance at 440 nm is often used as an index for the presence of antifermentative compounds and to assess the validity of a purification process (Egüés et al., 2013). The colour of the hydrolysates presented higher values in the trials with SSR 20:1, and the cultivar also seemed to affect this value, in particular with showed higher values for the white varieties than for the red ones (Table 2).

Table 2: Influence of the solvent to solid ratio and the cultivar on the colour of acid hydrolysates. The values followed by different superscript letters in the same column were statistically different.

Cultivar	Abs 440 nm	
	SSR 10:1	SSR 20:1
Chardonnay	0.71±0.01^{b}	0.75±0.01^{bc}
Moscato	0.66±0.06^{b}	1.75±0.01^{e}
Müller Thurgau	0.62±0.08^{b}	0.96±0.04^{d}
Barbera	0.44±0.01^{a}	0.59±0.01^{ab}
Nebbiolo	0.56±0.01^{ab}	0.48±0.01^{a}
Pinot noir	0.57±0.01^{ab}	0.80±0.02^{cd}

Regarding the hemicelluloses hydrolysis, the recovery of xylose (calculated based on the total used solvent volume) was lower compared with a previous paper (Spigno et al., 2013), with the highest yield for Chardonnay (Table 3). Also the correlation cultivar-sugar yield was not the same as found by Spigno et al. (2013), thus revealing a potential influence of the vintage. Glucose was always present (with the highest yield for Nebbiolo), confirming literature papers which reported glucose as an important constituent of stalks hemicelluloses (Prozil et al., 2012). A higher SSR did not enhance the hemicelluloses hydrolysis in almost all the cases, even though, the liquor concentration from SSR of 20:1 was about half that of the liquors obtained from a SSR of 10:1.

In white varieties, the concentration of TPC was slightly higher than in the red varieties, while the concentration of cinnamic acids was more related to the cultivar (Table 4). Absorbance at 320 nm used to estimate the cinnamic acids is also generally used to evaluate the soluble lignin. Both the parameters can indicate the presence of anti-fermentative compounds. Since the SSR of 20:1 led on an average to higher values, also for this aspect the SSR of 10:1 should be preferred.

Table 3: Influence of the solvent to solid ratio and the cultivar on sugars concentration in acid hydrolysates. The values followed by different superscript letters in the same column were statistically different.

Cultivar	Glucose g/L		$g_{glucose}$/100 g dry matter initial stalks	
	SSR 10:1	SSR 20:1	SSR 10:1	SSR 20:1
Chardonnay	3.68±0.06^{b}	1.86±0.01^{b}	1.38±0.01^{b}	2.04±0.06^{b}
Moscato	4.54±0.01^{c}	2.54±0.01^{c}	1.60±0.02^{c}	2.84±0.13^{c}
Müller Thurgau	6.55±0.01^{e}	3.48±0.04^{d}	2.52±0.04^{e}	4.14±0.07^{d}
Barbera	5.34±0.01^{d}	2.68±0.01^{c}	2.01±0.01^{d}	2.74±0.15^{c}
Nebbiolo	10.59±0.36^{f}	5.84±0.05^{e}	3.97±0.09^{f}	6.72±0.16^{e}
Pinot noir	3.06±0.15^{a}	1.22±0.08^{a}	1.07±0.09^{a}	1.30±0.07^{a}
Cultivar	Xylose g/L		g_{xylose}/100 g dry matter initial stalks	
	SSR 10:1	SSR 20:1	SSR 10:1	SSR 20:1
Chardonnay	7.42±0.35^{d}	3.63±0.24^{c}	5.68±0.29^{d}	5.65±0.24^{b}
Moscato	5.73±0.02^{b,c}	2.81±0.07^{b}	4.13±0.01^{b}	4.80±0.36^{a,b}
Müller Thurgau	6.21±0.27^{c}	2.95±0.16^{b}	5.03±0.23^{c}	5.49±0.68^{b}
Barbera	5.27±0.08^{b}	2.69±0.09^{b}	3.88±0.05^{b}	3.94±0.06^{a}
Nebbiolo	4.03±0.02^{a}	2.02±0.04^{a}	3.02±0.32^{a}	4.03±0.05^{a}
Pinot noir	4.16±0.09^{a}	2.05±0.11^{a}	2.95±0.08^{a}	4.24±0.04^{a}

Table 4: Influence of the solvent to solid ratio and cultivar on phenolics compounds in acid hydrolysates. The values followed by different superscript letters in the same column were statistically different.

Cultivar	Total phenols GAE-Folin mg/L		g/100 g dry matter initial stalks	
	SSR 10:1	SSR 20:1	SSR 10:1	SSR 20:1
Chardonnay	1197.87±55.62^{b}	868.60±3.36^{bc}	0.44±0.01^{bc}	0.95±0.03^{bc}
Moscato	1189.71±102.81^{b}	999.85±65.3^{c}	0.42±0.04^{abc}	1.11±0.10^{c}
Müller Thurgau	1215.20±54.89^{b}	797.45±31.38^{ab}	0.46±0.01^{c}	0.94±0.03^{bc}
Barbera	1096.10±37.74^{ab}	829.50±59.6^{bc}	0.41±0.01^{abc}	0.84±0.01^{ab}
Nebbiolo	850.40±113.36^{a}	635.30±40.41^{a}	0.31±0.03^{a}	0.73±0.05^{a}
Pinot noir	1018.95±89.82^{ab}	882.49±63.36^{bc}	0.35±0.04^{ab}	0.93±0.05^{bc}
Cultivar	Cinnamic acids FE-320 mg/L		g/100 g dry matter initial stalks	
	SSR 10:1	SSR 20:1	SSR 10:1	SSR 20:1
Chardonnay	103.49±0.27^{d}	87.82±1.08^{cd}	0.04±0.00^{cd}	0.10±0.01^{cd}
Moscato	100.21±1.86^{cd}	98.39±1.17^{d}	0.04±0.00^{cd}	0.11±0.01^{d}
Müller Thurgau	103.88±1.84^{d}	93.73±1.09^{d}	0.04±0.00^{d}	0.11±0.00^{d}
Barbera	65.71±2.95^{b}	59.33±1.42^{b}	0.03±0.00^{b}	0.06±0.00^{b}
Nebbiolo	91.66±4.89^{c}	71.36±0.31^{bc}	0.04±0.00^{c}	0.08±0.00^{c}
Pinot noir	23.71±1.48^{a}	22.26±0.29^{a}	0.01±0.00^{a}	0.02±0.00^{a}

Table 5 shows the SDC and phenolic acids detected in the hydrolysates. A higher SSR in the acid hydrolysis did not always lead to a significant higher hemicelluloses hydrolysis (based on xylose and glucose content). However, it caused an increase in phenolic compounds and SDC, confirming what was previously mentioned for the Folin index and cinnamic acids. SDC and phenolic acids showed a very high variability in replicates, resulting always in no significant differences among the cultivars. This may be due to the fact that the cooling phase of the hydrolysates was not tightly standardised (in terms of holding times in the switched off autoclave) and, in the case of phenolic acids also due to the very low concentration of this compounds in hydrolysates. Some compounds, as vanillin, syringaldehyde, sinapic acid, could be formed from a partial degradation of the lignin structure, and other could result from phenolic acids present in stalks; high values of gallic acid could be related from partial hydrolysis of tannins present in stalks. SDC concentration was very high, especially for 5-HMF and furfural, but 5-MF was detected in lower concentration. Therefore, a significant part of xylose and glucose were probably degraded during hydrolysis which resulted to be a too severe treatment.

Table 5: Influence of the solvent to solid ratio and the cultivar on sugar degradation compounds (5-HMF, furfural and 5-MF) and phenolic acids in acid hydrolysates. Data are expressed as mg/L.

	Barbera		Chardonnay		Moscato	
SSR	10:1	20:1	10:1	20:1	10:1	20:1
5-HMF	82.1±2.06	38.93±7.77	70.43±13.86	43.98±6.47	213.54±64.13	173.35±35.60
Furfural	147.61±48.58	112.41±7.43	162.54±47.12	132.62±26.74	138.54±56.55	65.6±7.42
5-MF	1.92±0.65	3.28±0.03	4.23±1.36	3.52±0.88	3.09±1.29	1.61±0.07
Gallic acid	54.47±13.44	37.5±2.07	37.81±5.37	26.46±1.90	37.13±1.58	26.11±4.39
Vanillic acid	0.57±0.22	0.45±0.25	0.77±0.12	0.64±0.17	0.29±0.11	0.18±0.08
Caffeic acid	0.16±0.07	0.17±0.07	0.19±0.08	0.18±0.08	0.09±0.03	0.04±0.01
Syringic acid	0.95±0.38	0.97±0.37	0.91±0.41	0.41±0.20	0.29±0.15	0.09±0.05
Vanillin	0.75±0.29	0.53±0.25	0.85±0.39	0.62±0.34	0.52±0.17	0.31±0.01
Syringaldehyde	0.88±0.37	0.52±0.23	1.02±0.44	0.74±0.36	0.47±0.09	0.36±0.02
Acetovanillone	0.05±0.02	0.08±0.02	0.05±0.02	0.04±0.01	0.15±0.05	0.20±0.02
Ferulic acid	0.08±0.04	0.07±0.02	0.04±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Sinapic acid	0.21±0.05	0.17±0.08	0.05±0.01	0.05±0.01	0.15±0.06	0.10±0.02
	Müller Thurgau		Nebbiolo		Pinot noir	
SSR	10:1	20:1	10:1	20:1	10:1	20:1
5-HMF	424.46±141.65	325.42±35.42	413.94±166.68	308.08±112.22	47.09±7.86	33.97±8.88
Furfural	107.88±0.32	91.51±2.66	141.67±57.42	98.25±35.71	97.49±46.66	80.51±39.31
5-MF	4.32±2.38	2.26±0.04	3.65±1.26	2.76±0.86	2.27±0.93	2.32±0.86
Gallic acid	42.29±10.72	27.34±7.14	40.7±5.96	29.23±4.00	43.74±0.07	28.82±2.06
Vanillic acid	0.70±0.17	0.77±0.34	0.49±0.21	0.42±0.15	0.60±0.28	0.49±0.15
Caffeic acid	0.13±0.06	0.13±0.04	0.21±0.07	0.03±0.01	0.06±0.02	0.05±0.02
Syringic acid	0.59±0.25	0.53±0.22	0.30±0.05	0.15±0.03	0.39±0.07	0.32±0.03
Vanillin	0.86±0.30	0.57±0.01	0.51±0.04	0.33±0.06	0.39±0.01	0.36±0.10
Syringaldehyde	0.64±0.29	0.46±0.03	0.53±0.24	0.24±0.03	0.28±0.05	0.30±0.01
Acetovanillone	0.08±0.01	0.16±0.02	0.16±0.01	0.06±0.01	0.03±0.01	0.04±0.01
Ferulic acid	0.08±0.02	0.07±0.03	0.10±0.03	0.06±0.02	0.04±0.01	0.04±0.01
Sinapic acid	0.17±0.05	0.20±0.03	0.16±0.07	0.15±0.04	0.12±0.01	0.12±0.02

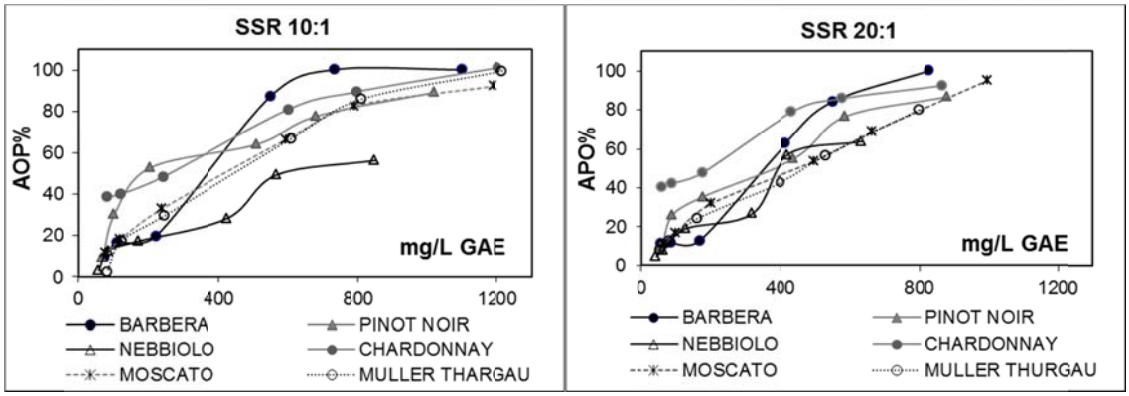

Figure 1: Influence of the solvent to solid ratio and the cultivar on the antioxidant activity (AOP%) of the acid hydrolysates as a function of total phenols concentration (GAE based on Folin index).

The ABTS test showed that the antioxidant activity of the hydrolysates was slightly influenced by the cultivar and substantially not by the SSR for the same cultivar (Figure 1). Mineral analysis was carried out only on the hydrolysates with SSR of 10:1 because this showed more convenient characteristics. Minerals, similarly to phenolic acids and SDC, presented high variability in replicates and did not present significant differences among the cultivar. Calcium and potassium were the most relevant cations detected in hydrolysates, while the other minerals were present in low concentration (Table 6).

Table 6: Influence of the cultivar on metals recovery in acid hydrolysates with SSR 10:1.

Variety	Barbera	Chardonnay	Moscato	Müller Thurgau	Nebbiolo	Pinot noir
Ca g/L	0.17±0.03	0.35±0.02	0.34±0.04	0.39±0.5	0.24±0.03	0.30±0.04
K g/L	1.83±0.41	1.55±0.29	1.10±0.24	1.12±0.19	1.26±0.28	1.35±0.29
Cu mg/L	0.17±0.07	0.18±0.8	0.05±0.01	0.03±0.01	0.08±0.02	0.05±0.02
Fe mg/L	0.17±0.02	0.17±0.03	0.10±0.02	0.11±0.02	0.11±0.01	0.10±0.02
Mg mg/L	24.70±11.29	23.21±10.28	29.01±13.33	30.69±0.38	15.83±5.20	22.24±9.21
Mn mg/L	0.04±0.01	0.10±0.01	0.05±0.01	0.08±0.02	0.07±0.01	0.19±0.03
Zn mg/L	0.05±0.01	0.10±0.02	0.06±0.01	0.04±0.01	0.05±0.01	0.05±0.02

4. Conclusions

A two-step process consisting in a water washing pre-treatment followed by a dilute acid hydrolysis has been proposed for the recovery of both non-structural and structural reducing sugars from grape stalks. Discrete amounts of glucose and fructose can be recovered in the pre-treatment which, however, needs to be optimised in order to get more concentrated solutions. The acid hydrolysis should be performed with a lower solvent-to-solid ratio (10:1 instead of 20:1) in order to reduce the formation of sugar degradation products that could limit the applicability of the liquors in fermentation processes. Moreover, the presence of phenolic acids and their related antioxidant activity could play an important antimicrobial role. On the other hand, mineral analysis showed, for all the investigated cultivars, a certain content in micro and macronutrients that could positively influence the microbial growth and metabolism. Sugars yield in the hydrolysis was influenced by the cultivar but with results not always in agreement with previous work, suggesting the possibility of using mixtures of stalks from different cultivars, which could greatly favour the industrial implementation of such a process.

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