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ARTICLE

Towards multi-purpose biorefinery platforms for the valorisation of agro-industrial wastes: production of polyphenols, volatile fatty acids, polyhydroxyalkanoates and biogas from red grape pomace

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The development of a multi-purpose four steps-cascading biorefinery scheme for the valorization of a red grape pomace (GP) was proposed. The consecutive processes were respectively dedicated to (a) the recovery of polyphenols by supercritical CO_2 extraction, (b) the production of volatile fatty acids (VFAs) by anaerobic acidogenic digestion, (c) the exploitation of produced VFAs as the precursors for the biotechnological production of polyhydroxyalkanoates (PHAs) and (d) the production of a CH_4-rich biogas by the anaerobic digestion of solid leftovers from the acidogenic process. More than 2.7 g of total polyphenols (as Gallic acid equivalents) per 100 g of dry matter were extracted. High contents of valuable proanthocyanidins occurred in the recovered polyphenolic fraction. The dephenolized GP was anaerobically digested under batch acidogenic conditions, obtaining about 20 g L^{-1} of total VFAs in the liquid effluent. The latter matrix was employed to feed a pure culture of a *Cupriavidus necator* strain, which was induced to produce and store PHAs under nitrogen-limiting conditions. The process was carried out in 0.5 L-shaken flasks by using a two-steps production approach. In particular, pre-grown biomass was fed with different concentrations of the acidic effluent (20 or 40% v/v) in two sequential batch processes. Poly(3-hydroxybutyrate) was accumulated up to 63% of the cells dry weight when pre-grown biomass was fed with 40% of the acidic effluent. No inhibitory effects due to non-VFA compounds occurring in the actual acidogenic effluent were observed. Finally, the anaerobic digestion of the exhausted solid fraction from the acidogenic process allowed obtaining 113 mL of biomethane per gram of fed volatile solids.

Introduction

According to an estimation reported by the OIV (International Organisation of Vine and Wine), 279 million hectolitres of wine were globally produced in 2014, 44.4 of which in Italy[1]. Winemaking processes lead to the generation of significant amount of solid and liquid residues. In particular, grape pomace (GP), which represents the main solid winery waste, consists of about 50% skins, 25% stems and 25% seeds[2]. Considering that 18 kg of GPs are averagely generated per 100 L of wine produced[3], about 5 million tons of such a residue are annually spawned worldwide, 0.8 of which in Italy. According to a previous regulation (EC Regulation 1493/99), GPs and lees winery wastes had to be processed by distilleries within the EU. Nowadays, a recent European reform of the wine sector (EC Regulation 479/2008) promotes the gradual withdrawal of distillation subsidies and consequently revokes the compulsory distillation. This should drive the promotion of integrated, sustainable and standardized alternative protocols for the valorisation of solid winery wastes[2].

In this frame, the development of multi-purpose cascading biorefinery schemes fed with GPs appears of great interest. This approach allows obtaining different valuable products by applying consecutive modular processes, along with a more extensive exploitation of organic leftovers, thus minimizing the generation of waste[4, 5].

The extraction of bioactive compounds from GP can represent an option for valorising the residue. In particular, GP polyphenolic compounds can exert beneficial effects on human health[6] and they were found in the grape skin and seeds after the fermentation process for the production of wine. Their extraction from GPs was already proposed for recovering highly valuable substances for the cosmetics, food additives (nutraceuticals) and pharmaceutical industries[5]. In particular, skins contain significant amounts of fibres (17-21%),

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fats (7-12%) tannins (16-27%) and other polyphenolic compounds (2-6.5%), among which catechins, anthocyanins, proanthocyanins, quercetin, ellagic acid and resveratrol. Seeds, in addition to oil, contain approximately 60% of the polyphenols occurring in grapes, with high concentrations of flavan-3-ols, catechin and epicatechin[2]. However, the proposed recovery subtracts only a minor organic fraction.

An alternative valorisation of GP could be represented by the production of a methane-rich biogas by anaerobic digestion (AD) processes[7]. However, low biomethanization performances were generally achieved. This was ascribed to the high content of lignin, which is not readily fermentable. Moreover, *Fabbri et al.*[8] reported the detection of a significant lag phase during methane production. Inhibition by alcohols and phenols was proposed among possible explanations. Some preliminary GP anaerobic digestion tests, confirming a scarce biomethanization of the waste, were carried out also in our labs. High volatile fatty acids (VFAs) production, and therefore their accumulation, was supposed as a further inhibitory cause[9]. On the other hand, VFAs, *i.e.*, linear short chain (C_2-C_6) carboxylic acids, are functional molecules, which can represent the precursors for the production of reduced added value chemicals (alcohols and aldehydes), polymers and biofuels in the frame of the carboxylate platform[10]. Thus, the acidogenic anaerobic digestion (AAD) of GP for the production of VFAs can be considered an alternative low-cost valuable approach for the valorisation of the biowaste.

Besides, VFAs are suitable precursors for the biotechnological production of polyhydroxyalkanoates (PHAs), which are microbial aliphatic polyesters naturally produced by many microorganisms. PHAs can exhibit similar or even better physicochemical properties with respect to those of petrol-based polyolefins[11-14]. Nowadays, PHAs are industrially produced by microbial pure cultures commonly fed with glucose[15]. Nevertheless, this approach hardly allows an economically competitive polymer production[16] if compared to that of petrol-based equivalent molecules, such as polypropylene. Alternative strategies based on the employment of mixed microbial culture (MMC) fed with VFA-rich effluents, which were obtained by digesting different biowastes under acidogenic conditions, were proposed with the aim of lowering the costs associated to the substrate and the process[17]. However, even the best results[18] showed that low PHAs concentrations can be obtained in MMCs effluents, and this negatively affects downstream costs. In addition to this, the employment of MMCs leads to a mixture of polymers instead of a well-defined single polymer type. Therefore, the development of new PHAs production processes mediated by pure cultures fed with VFAs-rich effluents appears of a great interest[19].

All this considered, the present work was dedicated to evaluate the technical feasibility of a multi-purpose cascading biorefinery scheme fed with a red GP for the obtainment of polyphenols, VFAs, PHAs and biomethane (**Fig. 1**). In particular, a supercritical CO_2 extraction (SC-CO_2) was applied for the recovery of polyphenols. The resulted dephenolised GP (GP_{Deph}) was anaerobically digested under batch acidogenic conditions for the production of a VFAs-rich liquid stream (GP_{Deph}^{Acid}). This liquid fraction was employed as the substrate for producing PHAs by a pure culture of *Cupriavidus necator*. Furthermore, the solid leftover from GP_{Deph}^{Acid} underwent a further methanogenic AD process dedicated to the production

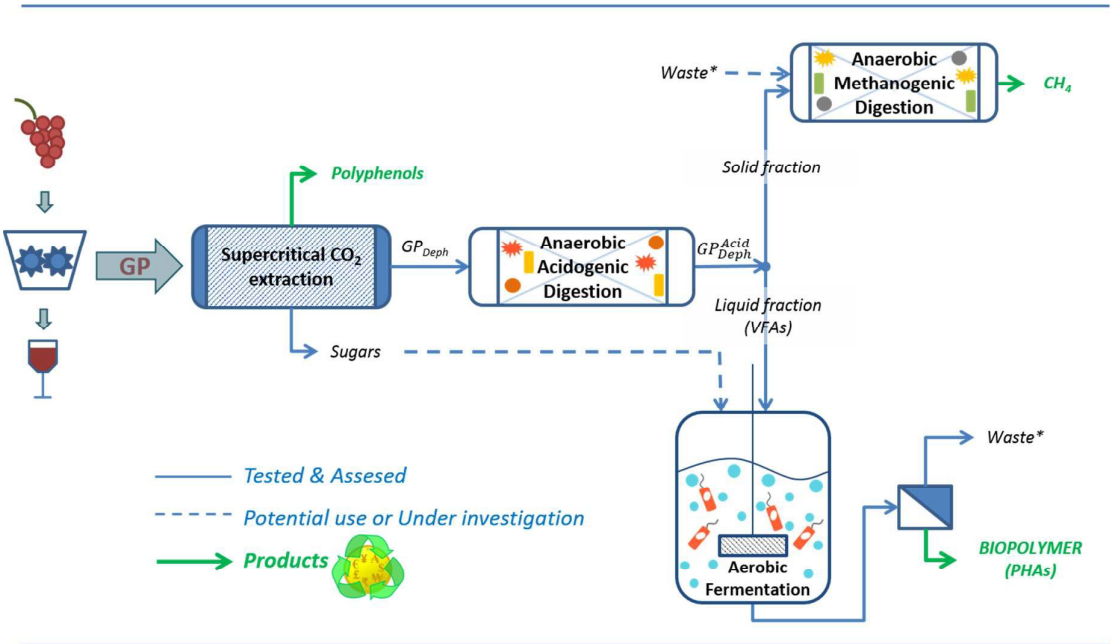

Figure 1: Multi-purpose biorefinery scheme for the obtainment of polyphenols and biopolymer from red grape pomace (GP).

of a methane-rich biogas.

To the very best of our knowledge, this is the first study dedicated to develop an integrated GP valorisation scheme, and, in particular, it represents the first attempt to produce PHAs with a pure culture of *C. necator* by employing digested GP as an alternative carbon source.

Results and discussion

Several approaches dedicated to the valorisation of grape pomace were reported in the literature, as reviewed by *Scoma et al.*[5]. However, most of these processes would hardly be economically feasible at an industrial scale if singularly applied. Conversely, multi-purpose integrated biorefinery could generate some positive synergistic effects, such as (a) costs investment optimization by better exploiting the diverse equipment, (b) diversification of the incoming profits by covering multiple markets/niches, (c) sharing manpower, (d) minimizing waste generation and (e) reaching the energy self-sufficiency (e.g.: biogas production from waste streams). This strategy could lead to an overall economical sustainability of the employment of a biowaste as an innovative renewable and low-cost feedstock[20]. In this frame, olive pomace was recently proposed as a raw material for the integrated production of natural antioxidants and renewable energy[21]. Moreover, the potential beneficial effects of multi-purpose biorefineries could be further enhanced if more than one waste is valorised. At the same time, this may also represent a solution for the valorisation of seasonal biowaste. As an example, the extraction of polyphenols from olive pomace and GP would allow the facility to run all over the year.

According to the mentioned strategy, the present work represents an attempt to evaluate the possibility of valorising a red GP by the integrated production of natural antioxidants, biopolymers and biogas. The four processes included in the proposed GP biorefinery scheme were studied separately and sequentially, in agreement with the cascade approach. Experiments were performed at bench-top/flask scale. Results are therefore presented according to the same processes sequence order.

Polyphenols extraction

The extraction of polyphenols from GP was studied by using: a) supercritical carbon dioxide (SC-CO_2) containing 10 % ethanol aqueous mixture at 57% (v/v) (SC-CO_2 +10% EtW) and b) conventional methanol extraction. The results for both methods are reported in Table 1. The process efficiency is quantitatively related to extraction yield. No statistically significant difference ($p \leq 0.01$) in the global yield of recovered dry matter (expressed as extracted mass per fed mass) obtained by SC-CO_2 and by methanol extractions was highlighted (Table 1).

The application of the SC-CO2 extraction allowed recovering 90% of the total polyphenols recovered within the conventional solvent method. The yield was higher than that reported by *Farías et al.*[22] (2200 mg_{GAE} 100 g_{DM}^{-1}), as well as the total antioxidant activity.

Table 1: Chemical composition of GP extracts obtained by methanol and SC-CO2 +10% EtW extraction methods.

	EXTRACTION METHODS	
	Methanol	**SC-CO_2 + 10% EtW**
Global Yield (% w/w)	15.6 ± 1.2 a*	14.6 ± 1.5 a
Total Phenols (mg $_{GAE}$ 100 g $^{-1}$ $_{DM}$)	2813 ± 10.8 a	2527 ± 11.5 b
Phenolic Yield (g $_{GAE}$ kg $^{-1}$ $_{extract}$)	180.3 ± 0.4 a	173.1 ± 0.5 b
Phenolic Yield (% SC-CO_2/methanolic yield)	100	90
Total Antioxidant Activity (mg $_{\alpha\text{-tocopherol}}$ 100 g $^{-1}$ $_{DM}$)	678 ± 15.5	8703 ± 17.5
Proanthocyanidins (mg $_{catechin}$ 100 g $^{-1}$ $_{DM}$)		
Monomeric fraction	1.2 ± 0.2	188.0 ± 3.8
Oligomeric fraction	4.1 ± 0.1	154.2 ± 5.8
Polymeric fraction	153.7 ± 0.2	361.5 ± 18.6
Antioxidant Activity (mg $_{\alpha\text{-tocopherol}}$ 100 g $^{-1}$ $_{DM}$)		
Monomeric fraction	28.1 ± 1.2	808.7 ± 10.2
Oligomeric fraction	30.1 ± 2.4	545.8 ± 7.3
Polymeric fraction	600.5 ± 2.9	3675.5 ± 6.8

Each data represent the mean of three replicates ± SD

* Values with different letter within row indicate significant differences ($p \leq 0.05$)

The obtained results indicate that the extracts recovered by the application of both methods contained a large number of soluble compounds, and that GP polyphenols included flavonoids and non-flavonoids[23]. Among the former ones, catechins and their oligomeric and polymeric forms, procyanidins (PCs), have been reported to exert potential health benefits in humans[24]. The healthy properties of catechins and PCs may depend on their structure and on their degree of polymerization. Monomeric structures have been shown to be quite efficiently absorbed, while oligomers reach the large intestine where they are efficiently converted into smaller metabolites by the local colonic microbial community[25]. In the present work, the amount of total catechin and PCs obtained by SC-CO2 was 703.7 mg of catechin equivalents per 100 g_{DM}^{-1}, and monomeric and oligomeric fractions together represented about half of total extracted flavor-3-ols. In particular, the small size oligomeric fraction was composed by several dimeric, trimeric and tetrameric B-types PCs (See ESI Table S1 and Fig. S1).

The SC-CO2 polyphenols extraction from GP was recently demonstrated to allow better performances with respect to those of a conventional solvent-based approach[2]. In fact, even if the total polyphenols extraction yields were nearly the same, the antioxidant activity was one order of magnitude higher when using the SC-CO_2. Yet more important, the SC-CO_2 extract presented a higher level of total proanthocyanidins (PAs) with monomeric and oligomeric fractions (Table 1). This suggests that supercritical CO_2 extraction of PAs from GP is more selective in extracting proanthocyanidins fractions - beneficial for human health- than methanol extraction. Finally, it is worth to note that about 60% of the total antioxidant activity resulted explained by PAs in SC-CO_2 +10% EtW, and 97% in the conventional extraction. This evidence, together with the previous observation indicate that the supercritical operating conditions developed are able to extract not only selectively the PAs, but also a great amount of other antioxidant compounds, not extractable with the conventional method.

VFAs production

After polyphenols extraction, the dephenolised leftover (GP_{Deph}) contained 90% of total solids (TS). Volatile solids (VS) were 90% of the later fraction. The application of a batch anaerobic acidogenic wet process onto such organic matter allowed the accumulation of a mixture of VFAs in the liquid phase. The VFAs concentration profile as a function of the experimental time is shown in **Fig.2**. The whole AAD lasted 16 days, after which 22.2 ± 0.8 g L^{-1} of total VFAs were obtained, this corresponding to 111 g of total VFAs per kilogram of GP_{Deph}. Among produced acids, acetic (15.5 g L^{-1}) and butyric (4.3 g L^{-1}) mainly accumulated in the medium. At the end of this process the measured COD of the dephenolised and acidified effluent (GP_{Deph}^{Acid}) was 35 ± 1 g COD L^{-1}. Since the COD due to the occurrence of VFAs (according to stoichiometric calculations) was 28.5 ± 1.5 g COD L^{-1}, more than 80% of the organic matter was represented by the target VFAs.

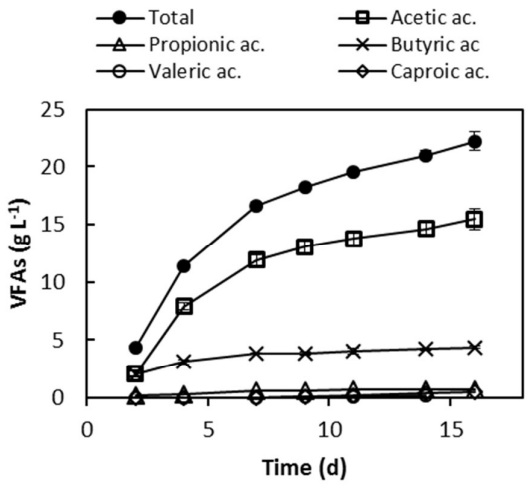

Figure 2: VFAs production from GP_{Deph}. Singles and total VFAs concentration trends.

The final VFAs concentration was comparable to that reported in a study where vinasse was used for VFAs production (19 g L^{-1} of total VFAs)[26]. Furthermore, comparable VFAs overall concentration was obtained when the same process was carried out using non-dephenolised GP as the substrate (about 23 g L^{-1}, See ESI Fig. S2). Taken together, such evidences seems to demonstrate that the preliminary polyphenols extraction process did not significantly lower the potentialities of the acidogenic step, probably both because a large availability of readily biodegradable organics still occurred in the GP_{Deph}^{Acid} and because the biological process is inhibited by higher overall VFAs concentrations.

9 mL g SV^{-1} of biogas were produced all along the anaerobic acidogenic digestion. Importantly, no VFA-consuming methanogenic activity was detected, while the overall produced biogas was composed by H_2 (35%) and CO_2 (65%). The total polyphenols content of the VFAs-rich liquid stream was 447 ± 39 mg L^{-1}.

PHAs production

Low cost substrates and high polymer amounts per cell dry weights are required in order to persecute the economic sustainability of biotechnological PHAs production. As a matter of fact, *C. necator* was found to grow and produce the biopolymer from diverse carbon sources [19, 27-32]. Among winery waste, wine lees were used as supplementary medium[33] and enzyme pre-treated GP (saccharified) were used as carbon source[34]. However, acidified pre-treated GP was never tested as the substrate for the biotechnological production of PHAs. Recently, an effective two-steps strategy for the production of PHAs from acidified olive mill wastewaters by *C. necator* was proposed[19]. In that work, the advantages of employing a two-stage production process (constituted by a preliminary balanced growth using glucose as the carbon source and a consecutive PHAs accumulation step under NH_4 limiting

conditions by feeding grown cells with VFAs) were discussed. Briefly, the employment of a low-cost alternative carbon source for the accumulation phase would allow replacing a large majority of the costly sugar required by the conventional PHA production process. In fact, PHAs may represent over 80% of the total CDW of *C. necator* strain[15, 35]. Hence, the same approach was applied in this work, where grown cells of *C. necator* were fed a) with different concentrations of the GP^{Acid}_{Deph} liquid fraction or b) with aqueous solutions containing the same amount of VFAs occurring in the mentioned experimental VFAs-rich substrates.

During all experiments, the preliminary growth phase lasted 24.5 hours. The final cell concentration was 2.5 ± 0.3 g L^{-1} and the glucose consumption was 5.0 ± 0.1 g L^{-1}. Thereafter, cells were harvested and re-suspended in the corresponding medium of each experimental test.

PHAs accumulation was observed for all conditions as linear increase of Abs_{600} (**Fig. 3A**). VFAs and PHAs profiles as a function of the experimental time are shown in **Fig. 3B** and **Fig. 3C**.

The complete consumption of the carbon sources was detected after 42 hours when GP^{Acid}_{Deph} represented 20% of the accumulation medium (**Fig. 3B**). Accordingly, a negative slope for biomass concentration, due to the consumption of accumulated PHAs (**Fig. 3C**), started to be observed after 42 h (**Fig. 3A**). Similar evidences were observed for the 40% conditions, since VFAs were not anymore detected after 44 hours and absorbance started to decrease two hours later (46 h). Therefore, cells were harvested and re-suspended in fresh media for the application of the second accumulation batch process, which lasted 46 hours in all experimental conditions. The 20% conditions were monitored until VFAs exhaustion, which occurred after a whole experimental time of 64 h (**Fig. 3B**). The 40% conditions were stopped after 70 h since no further significant absorbance increasing was detected. At that time, the overall VFAs concentration was 2 g L^{-1}. Final PHAs contents, PHAs yields, accumulation rates and final pH values are shown in **Table 2**. PHAs contents, which were measured according to GC analyses, were confirmed by TGA analyses (See ESI Fig. S3).

The highest PHAs content in cells fed with the actual VFAs-rich effluent (63%) was obtained for the 40% condition as a consequence of the application of the two consecutive

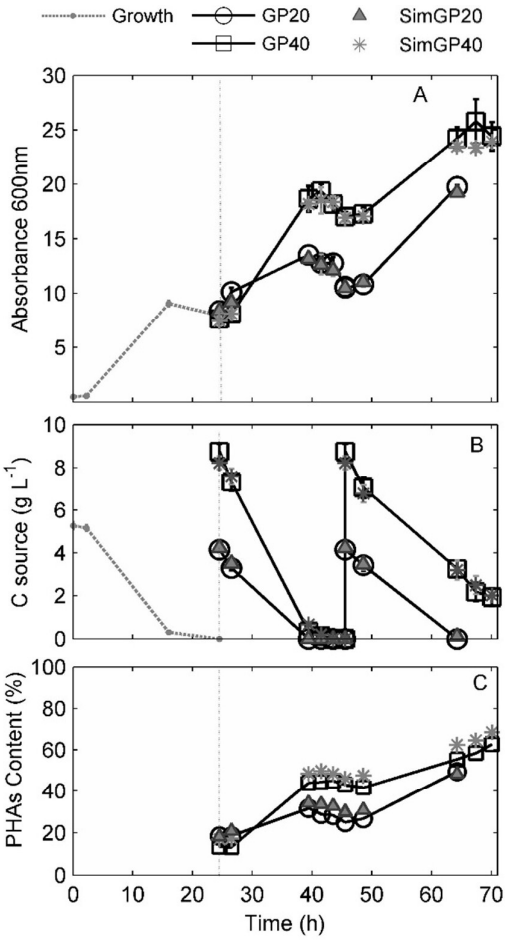

Figure 3: Responses of grown cells to 20% and 40% of GP^{Acid}_{Deph} and $SimGP^{Acid}_{Deph}$ contents in the accumulation media (% v/v). (A) Absorbance (Abs600) values as a function of the time related to growth (--●--) and accumulation phase. (B) Total VFAs consumption profiles. (C) PHAs content (% on a cell dry weight basis) obtained from GC analyses.

accumulation batch processes. This value represents an encouraging result for the design, set up and evaluation of the bioprocess at bench-top scale. Moreover, the application of a cell-recycling culture system, as demonstrated elsewhere[36, 37],

Table 2: PHAs content ($PHAs_{Cont}$); PHAs yield ($Y_{PHAs/VFAs}$); specific accumulation rate (Π_{Accum}); final pH values; obtained when using GP^{Acid}_{Deph} and $SimGP^{Acid}_{Deph}$ at different contents during the accumulation phase.

	GP^{Acid}_{Deph}		$SimGP^{Acid}_{Deph}$	
	20%	40%	20%	40%
$PHAs_{Cont}$ (%)	49 ± 1	63 ± 3	48 ± 1	68 ± 1
$Y_{PHAs/VFAs}$ (g PHAs g $VFAs^{-1}$)	0.26 ± 0.06	0.25 ± 0.04	0.26 ± 0.06	0.27 ± 0.05
Π_{Accum} (h^{-1})	0.0289 ± 0.0014[a] (0.0372 ± 0.0024)[b]	0.0645 ± 0.0019[a] (0.0211 ± 0.0032)[b]	0.0295 ± 0.0041[a] (0.0355 ± 0.0028)[b]	0.0607 ± 0.0035[a] (0.0204 ± 0.0009)[b]
ΔVFAs (g L^{-1})	8.29 ± 0.12	15.53 ± 0.13	8.37 ± 0.11	15.17 ± 0.12
pH_f	7.5 ± 0.1	7.9 ± 0.1	7.5 ± 0.1	8.0 ± 0.1

[a] Considering only the real accumulation time; [b] Calculated for the whole second phase duration

would allow a continuous feeding together with an increase of the final cell concentration.

The comparison among results related to the employment of the actual effluent and the VFAs solution suggests that no inhibition effects due to other organics in GP_{Deph}^{Acid} occurred. Indeed, GP_{Deph}^{Acid} tested concentrations were selected in order to avoid VFAs inhibition[38, 39], therefore it was important to exclude negative effects due to the effluent matrix. Polyphenols are well known anti-microbial agents. However, they probably did not inhibit the process both because of their low concentration in the GP_{Deph}^{Acid} (lower than the inhibitory concentration reported in a previous work[19]) and the fact that their antimicrobial activity is probably not significant for this case. This is in accordance with the wine fermentation process in which polyphenols do not cause inhibition.

The polymer production yields were lower than values previously published when pure acids were tested as the carbon source[40, 41] ($Y_{PHB/Acetic}$ = 0.47g g^{-1} and $Y_{PHB/Butyric}$ = 0.65 g g^{-1}, respectively). However, they were comparable to what obtained when a pre-treated olive mill wastewater was employed[19]. Furthermore, they resulted higher than yields reported when palm oil mill effluent and a pure culture of *Rhodobacter sphaeroides* (0.22 g PHAs g $VFAs^{-1}$)[42] or fermented organic waste and a pure culture of *R. eutropha* TF93 (0.16 g PHAs g VFAs-1) were used[43].

The lower calculated Π_{Accum} parameter related to both 20% conditions are coherent with previous studies[38, 39], reporting higher specific rates in response to a higher VFAs concentrations. This evidence was supposed to represent a kind of a mechanism for avoiding the toxic effects due to the acids.

On the other hand, the produced polymer was almost pure polyhydroxybutyrate (PHB). It is very well known that pure PHB has limited applicability, since it is highly crystalline and because its melting and degradation temperatures are closed each other[14, 44]. A possible perspective to persecute higher industrial interest for the proposed approach can be represented by the addition of a co-substrate such as propionic or valeric acids, these leading to the obtainment of the co-polymer poly (hydroxybutyrate-co-hydroxyvalerate), which is more flexible and stronger[44]. Propionic and valeric acids are VFAs that can be easily obtained from other biowastes or by modifying the AAD conditions[45, 46].

To identify whether PHAs were produced only from VFAs or from other compounds occurring in the complex GP_{Deph}^{Acid} matrix, too, initial and final COD values were measured and COD depletions were compared with theoretical calculated COD decays. The measured decreases of COD were 9.7 ± 2.4 gCOD L^{-1} and 15.4 ± 2.6 gCOD L^{-1} for 20% and 40% conditions, respectively. The calculated theoretical COD decays were 10.20 ± 0.15 gCOD L^{-1} and 19.65 ± 0.20 gCOD L^{-1}, respectively. These results suggested that other organics than VFAs did not significantly contribute to PHAs accumulation.

Biogas production from GP_{Deph}^{Acid} solid fraction

The net cumulative biogas production profiles as a function of the experimental time are presented in **Fig. 4**. A rapidly increasing cumulative CH_4 production was observed for about twelve days. After 31 days, 292 mL of biogas were produced. It was composed by methane (67.4%) and carbon dioxide (32.6%), while no hydrogen was detected. At the end of the experiment, 113 mL gVS^{-1} of biomethane where obtained.

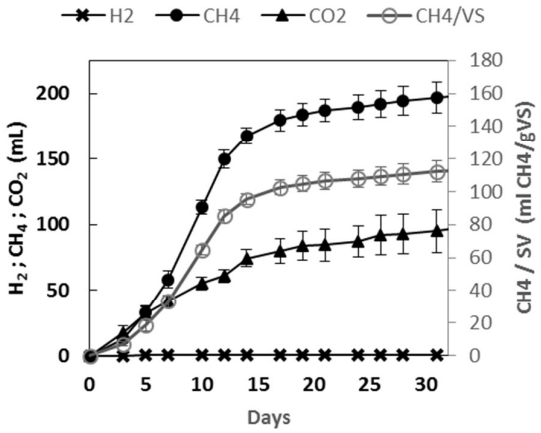

Figure 4: Effective biogas production using GP_{Deph}^{Acid} solid fraction. Accumulated hydrogen, methane and carbon dioxide production trends

Such a result did not represent a high biomethanization yield if compared to some evidences obtained with other biowastes[47]. Furthermore, the AD of the same non-pretreated GP at same inoculum to substrate ratio led to almost double biomethane production (data not shown). On the other hand, it was quite similar to the value reported by[7] also with non-pretreated GP and a lower inoculum to substrate ratio (0.66). The yields obtained by[8] were significantly higher than the obtained in the present work, but a shredding step was added for oil extraction from seeds. Therefore, the obtained results can be considered interest in the perspective of developing effective continuous anaerobic methanogenic processes fed with the target leftover and with the potentiality of also including the residues from the PHAs down-stream process.

Experimental

Chemicals and grape pomace

Folin-Ciocalteau reagent, gallic acid, (±) cathechin, (+)-α-tocopherol, vanillin 99%, the standard volatile fatty acids (VFAs) mixture (Supelco), poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (12 wt. % PHV; natural origin), salts (BioReagent) for the mineral medium, single VFAs and glucose (BioReagent) were purchased from Sigma Aldrich.

GP from red grape (*Vitis vinifera L.*) varieties was collected during September 2012 in Friuli Venezia-Giulia region (Italy). It was air dried at room temperature (moisture 14.3% ± 0.3 w/w) and stocked at 4°C until use. It was grinded with a domestic

miller, with an average particle diameter of 0.83 ± 0.05 mm as calculated with Sauter's equation[48].

Polyphenols extraction

The polyphenols recovery via supercritical CO_2 extraction was carried out using a commercial pilot-plant (SCF100 series 3 PLC-GR-DLMP, Separeco s.r.l, Pinerolo, Italy) equipped with 1 L extraction vessel, two 0.3 L separators in series and a tank for CO_2 storage. The gas was recycled after the separation process. A simplified flow sheet of the SFE pilot plant is given in **Fig. 5**.

Grinded GP was fed to the extractor (0.480 kg; density 600 kg m^{-3}) in order to be defatted by supercritical CO_2. As suggested by *Sovová et al.*[49], pressure was 28 MPa and temperature 45°C, while CO_2 flow rate was 10 kg h^{-1} and the total extraction time was 3 h. Such conditions corresponded to 62.5 Q (kg CO_2 kg $feed^{-1}$). Subsequently, a co-solvent was required for extracting polyphenols from the defatted GP, due to the polarity of polyphenols. Therefore, 0.1 kg of defatted GP were treated with supercritical CO_2 containing 10% ethanol–water mixture (57%, v/v) (EtW) as co-solvent at 8 MPa, 40°C and CO_2 flow rate of 6 kg h^{-1} [2]. Aliquots of grape extract were collected during extractions in volumetric flask at intervals of about 30 min, to asses several data points for the overall extraction curves (OECs). The ethanol aqueous mixture was then removed from the extracts with rotary evaporator (Buchi, B465, -Switzerland) at 45°C. After solvent removal, extracts were weighted and analysed. All experiments were conducted in duplicate. The statistical significances of the differences between means were determined using Tukey's test with the level of significance set up at p≤0.05.

Anaerobic acidogenic digestion

The anaerobic process was inoculated with an acidogenic microbial consortium, which was obtained from an anaerobic treatment of organic fraction of municipal solid waste and acclimated to the acidogenic digestion of GP and exhausted in terms of VFAs production. The GP_{Deph} coming from the extraction step was characterized in terms of total solid content (g TS g GP_{Deph}^{-1}) and volatile solids content (g VS g TS^{-1}). Thereafter, a 1 L-Pirex bottle (supplied with a tri-ports cap with silicone septum) was fed with water, GP_{Deph} and a microbial inoculum (10%, v/v), so that final working volume and TS content were 560 mL and 20% (w/v), respectively. Incubation conditions were: pH 7, 37ºC and 150 rpm. The process was monitored every 2-3 days for biogas and VFA productions. To the latter aim, 500 µL-liquid samples were withdrawn. pH was corrected to 7 by the additions of NaOH 10 M after each monitoring process. During such operations, nitrogen was flushed to maintain anaerobic condition. VS were determined at the end of the digestion in order to evaluate the amount of organic matter consumed during this step. The experiment was carried out in triplicate.

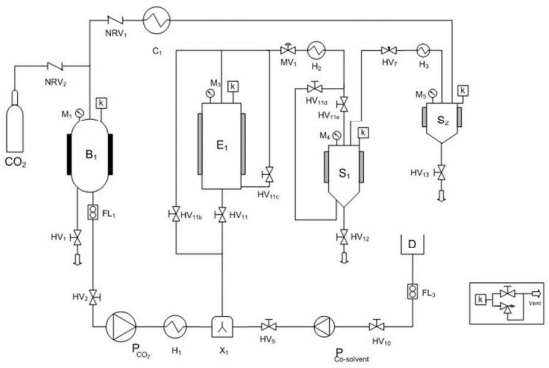

Figure 5: Schematic pilot-scale extractor: (B1) Storage tank, (E1) extraction vessel, (S1, S2) separators, (H#) heat exchangers; (C1) condenser; (HV#) Hand valves; (MV1) membrane valve; (NVR#) no return valves; (P) diaphragm pumps; (F1) flowmeter; (M#) manometers; (k) safety devices; (FL1) coriolis mass flowmeter; (D) co-solvent storage tank; (X#) mixer

PHAs production

Bacteria strain, inoculum and culture media. *Cupriavidus necator* (DSMZ 545) pre-culture was started from agar plates and grown within 24 hours in a 500 mL-Erlenmeyer flask containing 150 mL of LB; incubation conditions were 30°C and 150 rpm.[19] The experiments were performed according to a dual-phase process (reported above). In brief, PHA accumulation was induced after a preliminary phase, during which cells were grown under optimal conditions. A slightly modified Medium 81 from DSMZ was employed for the cell balanced growth (growth phase); it contained 3 instead of 1 g L^{-1} of $(NH_4)_2SO_4$. Glucose (5 g L^{-1}) was added as the sole carbon source**.** Conversely, an ammonia free-medium was employed for the subsequent PHAs accumulation phase. It was prepared by combining two sterilized stock solutions, namely: (a) the VFAs-rich effluent obtained by the acidogenic digestion of GP_{Deph} $\left(\boldsymbol{GP_{Acid}^{Deph}}\right)$, which was filtered (Whatman N11, 11µm), amended with Medium 81-DSMZ salts (except for $(NH_4)_2SO_4$) and autoclaved using special Beckman flasks allowing a subsequent centrifugation (8000 rpm, 4ºC and 25 minutes) under sterile conditions; and (b) distilled water amended with Medium81-DSMZ salts (except for $(NH_4)_2SO_4$) at the same concentrations they occur in such a medium. The accumulation culture media were prepared by mixing the two stocks solutions at different proportions, namely: 20 and 40% v/v of $\boldsymbol{GP_{Acid}^{Deph}}$. In addition to this, a parallel control test was carried out using a simulated $\boldsymbol{GP_{Acid}^{Deph}}$ $\left(\boldsymbol{SimGP_{Acid}^{Deph}}\right)$, which was a VFAs solution prepared by dissolving in distilled water the organic acids at the same concentrations relieved in $\boldsymbol{GP_{Acid}^{Deph}}$. The control test was aimed at verifying whether other compounds than VFAs occurring in $\boldsymbol{GP_{Acid}^{Deph}}$ could affect PHAs accumulation. Two sequential accumulation batch processes were carried out in all conditions with an initial pH of 7.2.

Experimental approach. The whole PHAs production process was separated in a two-stage batch cultivation procedure. A growth phase (1st process phase) was carried out under balanced growth conditions, using glucose as the carbon source, and it was started by inoculating 500 mL-Erlenmeyer flasks containing 150 mL of the growing culture media. To this aim, pre-grown cells were harvested by centrifugation (6000 rpm for 5 minutes at 4°C) and suspended in the media to an initial absorbance (Abs600) of 0.4. The incubation conditions were the same previously mentioned. After 24 hours, the growth phase was concluded and cells were harvested by centrifugation (6000 rpm for 5 minutes at 4°C). Thereafter, the grown biomass was re-suspended in the experimental accumulation medium at the same concentration they occurred at the end of the growth phase, this representing the beginning of the subsequent PHAs accumulation phase (2nd process phase).

In this way, the possibility of using $\boldsymbol{GP}_{\boldsymbol{Acid}}^{\boldsymbol{Deph}}$ as an alternative carbon source specifically only for PHAs production was studied. The latter acid effluent constituted 20% and 40% of the accumulation phase media, as reported previously, in order to determine if $\boldsymbol{GP}_{\boldsymbol{Acid}}^{\boldsymbol{Deph}}$ concentration could affect the PHAs accumulation activity of grown cells. Each experiment was carried out in triplicate.

Biogas production

The solid leftover from the anaerobic acidogenic digestion step $\left(GP_{Acid;Solid}^{Deph}\right)$ was tested as a substrate for biogas production. The experiments were carried out in 100-mL Pyrex bottles (microcosms, 55 mL of working volume) tightly closed with a modified Pyrex-cap that allowed gas sampling. The inoculum to substrate ratio was 1 g of VS in the inoculum per g VS in the substrate, and the TS content was 8% (92% of which VS). The methanogenic microbial consortium employed as inoculum was obtained from a commercial biogas production plant located in the Emilia Romagna Region (Italy) fed with agro-industrial biowastes and zootechnical liquor. It was exhausted in terms of gas production before being employed. Incubation conditions were 37°C and 150 rpm. The experiment was carried out in triplicate. A blank control experiment was set up by filling microcosms only with water and the inoculum, in order to calculate the effective biogas production by subtracting the amount of biogas eventually produced within control experiments to that produced within target test. Biogas production was measured every 2-3 days. After biogas sampling, the bottles were opened under nitrogen gas flux to keep anaerobiose and pH was corrected to 7.5 by adding few drops of H_2SO_4 10 M. All the adopted experimental conditions were recommended by[47, 50].

Analytical procedures

Polyphenols extraction. All procedure were carried out as previously described[2].

The total phenolic content (TPC) of the extracts were measured using Folin-Ciocalteau reagent, according to *Yu et al.*[51]. A calibration curve was made with standard solutions of gallic acid in the range 0.2–10 mg mL^{-1} and measures were carried out at 765 nm (R^2= 0.99). Results were expressed as milligrams of equivalent gallic acid per 100 gram of dried matter ($mg_{GAE}\ 100\ g_{DM}^{-1}$).

The fractionation of proanthocyanidins from the extracts was done as reported by[52], as well as the total flavan-3-ol content that was determined by vanillin assay. Results were expressed as milligrams of equivalent catechin acid per 100 g of dried matter ($mg_{catechin}\ 100\ g_{DM}{}^{-1}$).

The antioxidant activity of phenolic extracts and proanthocyanidins fraction was evaluated by the total free radical scavenger capacity (RSC) following the methodology described by[53] with slight modification[2]. The antioxidant activity of the samples was expressed as the milligrams of α-tocopherol per 100 g of dried matter $\left(mg_{\alpha-\text{tocopherol}}\ 100\ g_{DM}^{-1}\right)$. A calibration curve was made with standard solutions of α-tocopherol in the range 5.8×10^{-5} – 2.3×10^{-3} mol L^{-1} (R^2= 0.98).

All analyses were performed in triplicate.

The qualitative characterization of polyphenolic extracts was carried out by UHPLC-MS^n analyses as reported by *Bresciani et al.*[54]

VFAs determination. VFAs concentration were determined by GC-FID analysis (Agilent 7890A). A HP-INNOWAX column (ID 0.25 mm, length 30 m and film thickness 0.25 µm) was employed under the following conditions: injector and FID temperature were 250 °C and 280 °C, respectively; pressure was 9.5649 psi; H_2 flow was 30 mL min^{-1}; air flow was 300 mL min^{-1}; carrier gas flow rate (nitrogen) was 29.281 mL min^{-1}, with a split ratio of 10:1 (7 mL min^{-1}); injection volume was 1µL. The temperature programme was: 80 °C for 0.5 min, then 20 °C min^{-1} to 150 °C for 1 min, then 20 °C min^{-1} to 240 °C for 2.5 min. Before the analyses, the samples were diluted with an equal amount of a 60 mM oxalic acid solution.

At the end of the fermentation, organic matter content in the liquid phase was measured by determining chemical oxygen demand (COD) of the samples supernatant experimentally and theoretically, therefore obtaining the percentage of the total COD content that was ascribed to the occurrence of VFAs $\left(\frac{COD_{VFAs}}{COD_{TOTAL}} * 100\%\right)$.

TPC in the $\boldsymbol{GP}_{\boldsymbol{Deph}}^{\boldsymbol{Acid}}$ was measured by colorimetry with a down-scaled procedure of the method reported elsewhere[55].

Chemical oxygen demand (COD). A colorimetric commercial kit (AQUALYTIC Vario MR) was used. At the same time a theoretical COD was calculated by only taking into account the VFAs oxidation: concentrations were expressed as COD equivalents according to stoichiometric calculations.

PHAs production. Sampling was performed periodically. The procedures for sample treatment and analysis were the same previously described in[19].

When performing the Abs600 vs. cell dry weight (CDW) calibration curve, linear correlations were obtained for the growth and the accumulation phases (data not shown). PHAs content was defined as g PHAs . g CDW^{-1} . 100%, on a cell dry weight basis.
Organic matter consumption during the accumulation phase was followed by measuring the samples supernatant COD and the theoretical COD variation was calculated.

Biogas production. Production of biogas was measured in terms of volume (glass syringe) and composition. This last, in terms of H_2, O_2, CH_4 and CO_2, was measured by gas-chromatography using a μGC (model 3000 A - Agilent Technologies, Milano, Italy) under the following conditions: injector temperature 90 °C; column temperature 60 °C; sampling time 20 s; injection time 50 ms; column pressure 25 psi; run time is 44 s and the carrier gas was nitrogen.
TS were determined by conventional gravimetric method exposing the sample to 105°C overnight and VS were determined by exposing the resulted dried sample to 600°C for 1 hour.

Conclusions

In conclusion, the possibility of developing a multi-purpose biorefinery scheme for the valorisation of a red grape pomace by the obtainment of natural antioxidants, volatile fatty acids, biopolymers and biomethane was demonstrated. The extracted polyphenolic fraction included significant amounts of bioactive compounds, which are readily adsorbed by the organism. The acidification of the dephenolised residue was obtained by feeding the organic matrix to a biological anaerobic acidogenic process. The resulting VFAs-rich liquid effluent was employed as the substrate for an effective biotechnological production of PHAs. Biomethane was obtained from the exhausted solid leftover, which was digested under anaerobic methanogenic conditions. To the very best of our knowledge, this study represents the first attempt of exploiting grape pomace for the integrated production of several industrial products. In particular, the target biowaste have never been tested before as an alternative low-cost substrate for the production of PHAs.

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