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Upgrading grape pomace contained ethanol into hexanoic acid, fuel additives and a sticky polyhydroxyalkanoate: an effective alternative to ethanol distillation†

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The management of grape pomace (GP), the main winery solid residue, is presently supported by government subsidies, promoting the energetically expensive recovery of ethanol by distillation. This work proposes and assesses a novel sustainable alternative GP valorisation strategy: chain elongation fermentation. Besides, the proof-of-concept of a multipurpose cascading scheme is presented based on experimental data for each step on the laboratory scale. The new cascading biorefinery scheme includes: (1) the ethanol upgrade into highly concentrated (*ca.* 900 g L^{-1}) *n*-hexanoic acid (C6) by anaerobic acidogenic fermentation and a simple downstream; the exploitation of the obtained C6 as (2) a reagent for obtaining an ester-alcohol mixture as well as (3) a substrate for the production of medium chain length polyhydroxylalkanoates (mcl-PHAs), and (4) the complementary biomethanization of the solids leftovers from the acidogenic step. Specifically, the identified fermentation conditions (pH 7, 37 °C and $P > P_{atm}$) allowed obtaining the highest C6 titer (22 g L^{-1}) and productivity (6.2 g L^{-1} d^{-1}) ever achieved from non pre-treated biowaste and without the need of either exogenus ethanol or methanization inhibitor or expensive in-line extraction methods. Such titer, allowed employing a cheap easy-direct C6 downstream leading to 54% C6 recovery (87% purity) at potentially competitive overall costs. Although a preliminary assessment showed that this partial valorisation could be economically sustainable in itself for GP-management, the exploitation of the highly concentrated GP-derived C6 was demonstrated for the first time. Heterogenous catalytic hydrogenation (180 °C and 115 bar), with pre-reduced commercial catalyst Re/C 5 wt%, allowed the conversion of the obtained C6 into a mixture of 1-hexanol and hexyl-hexanoate (molar yield of 75%), which represents a promising blendstock for both diesel and biodiesel fuels. On the other hand, a fed-batch culture system, carried out on a bench-top bioreactor, allowed obtaining 60% PHA content with a yield of 0.30 g g^{-1}. The recovered sticky bioelastomer was mainly composed of 3-hydroxyhexanoate (90%). Complementarily, *ca.* 200 N-L $kgVS^{-1}$ of biomethane was obtained from GP leftover solids.

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Introduction

Grape pomace (GP) is an agro-industrial by-product generated during the winemaking process. Specifically, GPs are the solids separated (i) after crushing and pressing grapes but before vinification (white wine) or (ii) after vinification (red wine). Thus, GP from red wines contains more ethanol and less sugars than GP from white wines.[1,2] According to the report from the International Organisation of Vine and Wine, 57% of the world grape production (77.8 Mt per year, in the year 2018) is used for making wines (292 MhL per year, in the year 2018).[3] Therefore, considering GP as the grape material which is not converted into wine, the GP generation ratio can be estimated to be 0.25–0.34 kg_{GP} $kg_{fresh\ grapes}^{-1}$, which is slightly higher than the ratios previously reported by Rockenbach *et al.*[4] (0.18 kg_{GP} L_{wine}^{-1}) and Van Dyk *et al.*[5] (0.20 kg_{GP} $kg_{fresh\ grapes}^{-1}$). Hence, more than 9 Mt of GPs (red and white) are generated worldwide annually, 18% of which

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comes from Italy (*ca.* 50% from red and 50% from white wine productions). GPs are recognised as potential environmental pollutants that must be treated before discharge.

Multipurpose integrated biorefineries are crucial for the biological cycles of a circular economy since they allow valorisation of the various biomass fractions by applying different cascades in an economically sustainable way. Indeed, there are already GP biorefineries around the world, where ethanol (for fuels additive or pharma, among other applications), anthocyanins and tartaric acid are recovered as products.[6] Despite the fact that ethanol recovery by distillation is a subsidised economic practice,[7,8] still represents the main final destination of GP in Italy, France and Spain.[6] Although this policy is environmentally sustainable, the development of a techno-economically feasible alternative would allow achieving both the environmental and the economic goals in GP management.

In this line, a novel integrated scheme for the valorisation of red GP was assessed for the first time in a previous study (Fig. 1).[9] It allowed the recovery of polyphenols (antioxidant compounds) from GP and obtaining short chain length polyhydroxyalkanoates (scl-PHAs; biopolymer), as well as CH_4-rich biogas from the dephenolised and fermented GP (GP_{Deph} and GP^{Acid}_{Deph}), respectively. Despite its technical viability, the economic feasibility of the scl-PHA production depends on the application of either (i) perfusion culture system allowing continuous feeding of relatively low concentrated short chain length carboxylates (SCCs) and purging the exhausted broth[10,11] or (ii) a SCC concentration step[12] for obtaining a suitable high titer feeding solution to carry out the fed-batch culture. Moreover, ethanol valorisation was not assessed in that scheme since it was removed by the polyphenol extraction process.

Hence, an alternative ethanol valorisation route is proposed by modifying our previously reported scheme, *i.e.*: by-passing the polyphenol extraction step so that the ethanol contained in red GP could be upgraded into medium chain length carboxylic acids (MCCs; containing 6 to 12 carbons) by directly fermenting fresh red GP (GP_{Fresh}) anaerobically (Fig. 1, red dashed arrows). Briefly, chain elongation anaerobic fermentation using open culture technology (microbial consortia) allows microbes to transform a substrate (*i.e.* electron donors like ethanol or lactate) into MCCs through the reverse β oxidation pathways.[13] MCCs are of major industrial interest since they can be used directly as antimicrobial agents in agriculture or transformed into flavours and fragrances, lubricants, rubbers, dyes and renewable diesel or aviation fuel.[13–17] Actually, MCCs are produced from natural fats and oils (*e.g.* coconut oil for hexanoic acid) using physicochemical processes which are more complex and expensive than simple anaerobic fermentation, namely: (1) chemical or high pressure hydrolysis (255 °C and 49 atm.), (2) solvent crystallisation, (3) hydrogenation and (4) distillation.[18] Moreover, such an alternative route to ethanol valorisation can lead to some advantages, which include (i) saving high costs associated with ethanol recovery by distillation (5265 kJ L^{-1}, ~22% of its combustion energy);[19] (ii) obtainment of higher added value products (*i.e.* MCCs, biofuels or PHAs); and (iii) production of compounds with a lower oxygen/carbon ratio (*e.g.* *n*-hexanoic acid has a mass ratio of 0.44 as compared to ethanol's 0.67, and thus a higher energy density, notably 28789 kJ L^{-1} that exceeds that of ethanol by ~22%).

To the best of our knowledge, the production of MCCs from GP has never been reported, whereas it has been studied from wine lees,[20] liquor-making effluents[21] or other agro-industrial by-products.[15,17] Such an unexplored GP valorisation strategy would allow overcoming the economic issues mentioned above, including those for the scl-PHA production in the previously reported scheme, since: (1) MCCs have low water solubility, thus requires less energy expenditure downstream for obtaining highly concentrated single MCCs; (2) the concentrated MCCs represent an optimal alternative reagent for the production of esters and alcohols *via* catalytic-hydrogenation as well as a substrate for the production of medium chain

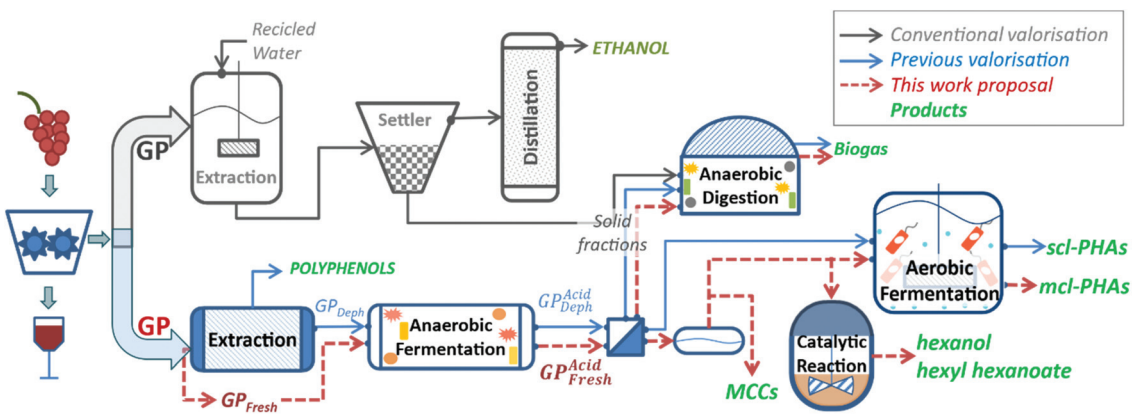

Fig. 1 A conventional GP biorefinery scheme for ethanol recovery (in grey) towards a GP valorisation strategy (in colours). The feasibility of polyphenols recovery and SCC production was previously demonstrated (blue arrows). The present work proposes to by-pass the extraction step (red dashed arrows) for upgrading the contained ethanol to MCCs and these into biofuels and mcl-PHAs.

length PHAs (mcl-PHAs) by applying fed-batch culture systems that allow obtaining a high cell density. The mcl-PHAs are bio-elastomers with different applications, *e.g.* tissues, drug delivery, adhesives, among others.[22]

Previous studies on MCC catalytic hydrogenation were carried out using commercial pure hexanoic or octanoic acids as reagents.[23] The hydrogenation of lower butyric acid from fermentation was studied in the gas phase at 265 °C and 25 bar of H_2 in the presence of a ZnO-supported Ru–Sn bimetallic catalyst.[24] Importantly, the employed butyric acid was not the raw fermentation product, as proposed in this work, but it had been recovered from the fermentation broth by solvent extraction and further purification by reduced pressure distillation. Moreover, the adopted drastic conditions and the use of *ad hoc* synthesised ruthenium catalyst represents a potential barrier for large-scale applications. Differently, the transformation of MCC into biofuels has recently been assessed by using the actual biotechnologically produced MCC-mixture adopting an alternative approach. The process included a solvent extraction step (herein avoided) and Kolbe-electrolysis which allowed obtaining alkanes (drop-in fuels) while ester and alcohols represented the by-products.[25]

Regarding the mcl-PHA production from actual agro-industrial by-products, reported studies included intensive pre-treatments such as (i) chemical/enzymatic saccharification,[26] (ii) anaerobic fermentation followed by solvent extraction[27] or (iii) chemical hydrolysis of exhausted cooking oil.[28] Differently, the production using MCCs would involve both less intensive or sophisticated pre-treatments and allow modulating the monomer composition (*i.e.* single acids could be fed).

Therefore, the present work was dedicated to assessing the technical feasibility and optimisation of directly fermenting red GP_{Fresh} for producing MCCs. Such a step could (i) prove to be an alternative to the conventional ethanol recovery by distillation or (ii) be used to enlarge the portfolio of red GP derived products. Regarding polyphenol extraction, the new proposed cascade scheme (Fig. 1, red arrows) does not include it as it has been already studied, however, it could be carried out in a parallel process. Thus, in this work screening tests were carried out for the anaerobic fermentation of GP_{Fresh} to find the optimal temperature, pH and pressure that maximise the of MCCs. Thereafter, GP_{Fresh} was anaerobically fermented under optimal conditions to produce high quantities of a hexanoic acid rich liquid (GP^{Acid}_{Fresh}). This allowed us to verify the feasibility of applying a simple downstream procedure for obtaining a highly concentrated product and to perform a preliminary technoeconomic evaluation of the valorisation route which was compared with the conventional ethanol distillation. Moreover, the proof-of-concept of a cascading multi-purpose biorefinery scheme is presented (Fig. 1) based on experimental results obtained with the actual highly concentrated hexanoic acid subsequently tested as (i) a reagent for producing a mixture of hexyl-hexanoate and hexanol by means of a catalytic reaction and (ii) as a substrate for producing mcl-PHAs by a pure culture of *Pseudomonas putida*. Additionally, the solid leftover from GP^{Acid}_{Fresh} underwent a further anaerobic digestion process dedicated to the production of methane-rich biogas. To the very best of our knowledge, this is the first study reporting an alternative to ethanol valorisation by distillation of GP_{Fresh}, and it represents the first attempt to produce chemicals and mcl-PHAs from highly concentrated hexanoic acid derived from agro-industrial by-products such as GP_{Fresh}.

Results and discussion

The four intended processes were studied sequentially, in agreement with the cascade approach. Experiments were performed at flask/bench-top scale and the results are presented according to the proposed scheme sequence order.

Production of hexanoate from GP_{Fresh}

Screening of operating conditions. GP_{Fresh}, directly arising from the wine making process, was anaerobically fermented in the batch mode. All tested conditions had initial ethanol and total solid concentrations of 20.8 ± 0.7 g L^{-1} and 148.2 ± 4.7 g L^{-1}, respectively. Fig. 2 shows the concentration profiles of main metabolites during the batch tests for different pH conditions at 37 °C until day 13. It can be observed that *n*-hexanoate (C6) started to be detected when *n*-butyrate (C4) concentration became >2 g L^{-1}. This trend is in accordance with the chain elongation mechanism (*i.e.* firstly produced and accumulated acetate is used as a substrate for the consecutive elongation to butyrate and caproate) and perfectly in agreement with the results recently published by San-Valero *et al.* related experiments carried out with a pure culture of *Clostridium kluyveri* fed with commercial acetic acid and ethanol.[13,29] In the same work San-Valero *et al.* reported immediate hexanoic acid production when butyric acid was fed from the beginning (>5 g L^{-1}) together with ethanol and the rate decreased when the butyric acid concentration was <2.5 g L^{-1}. Hence, from the two initially considered potential reasons (thermodynamic and kinetic), it was inferred that it is a matter of kinetics: hexanoic acid production rate becomes significant when butyric acid is ≤2.5 g L^{-1} later the rate decreased but the reaction was still exergonic for continuous producing. The highest C6 concentration and total carboxylate titer were obtained at pH 7 (Fig. 2C): 20.2 ± 0.2 g L^{-1} and 29.4 ± 0.7 g L^{-1}, respectively. Carboxylates represented 85 ± 2% of the total soluble COD, *i.e.* anaerobic fermentation is highly acidogenic. The attained higher concentrations were in accordance with the previous evidence suggesting that pH neutrality avoids inhibition by undissociated acids ($pH \geq pK_a + 2$).[16] Unlike the SCC mixture obtained in the previous study[9] by the fermentation of GP_{Deph} also at pH 7, the GP^{Acid}_{Fresh} blend was mainly composed of *n*-hexanoate (44%), acetate (25%) and butyrate (23%). Furthermore, the C6 concentration attained in this work represents the highest titer obtained by anaerobically fermenting an untreated biowaste.[15] The obtainment of a high concentration of MCCs represents important evidence from the perspective of separating the carboxylates by directly diminishing the pH of the reactor effluent ($pH \leq pK_a - 2$).[30]

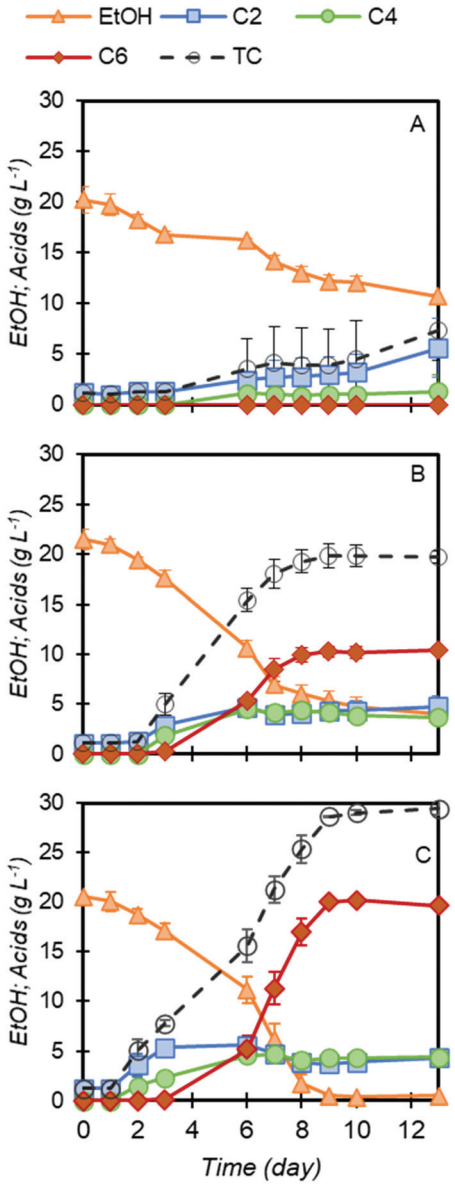

Fig. 2 Microbiome responses to different pH conditions, namely: 5 (A), 6 (B) and 7 (C). Metabolite concentration profiles (ethanol, EtOH; acetic acid, C2; butyric acid, C4; hexanoic acid, C6 acids; total carboxylates, TC) during the batch fermentation at 37 °C.

Only two previous studies reported higher C6 concentrations (27.55 and 31.5 g L^{-1}) when using actual agro-industrial by-products such as willow wood digestion liquor and organic municipal waste.[13,31] However, those substrates were pre-treated and exogenous ethanol (commercial ethanol) was used for the chain elongation; both requiring additional costs. Instead, GP_{Fresh} already contains ethanol and did not require any thermal or chemical hydrolysis pre-treatment.

On the other hand, no hexanoate production was observed under any condition at 55 °C (ESI, Fig. S2A, B and C†). Considering that temperature does not significantly affect the ΔG of chain elongation reactions, it was assigned to the fact that optimal temperature for target microorganisms (*e.g. Clostridium kluyveri*) in the actual consortium is below 40 °C.[15]

Furthermore, pressure is an important operating parameter. Indeed, the final hexanoate concentration decreased to 40% when anaerobic fermentation was carried out under the optimal conditions (pH 7 and 37 °C) but using a dynamic headspace volume which allowed working under almost constant atmospheric pressure (see ESI Fig. S4 and S5†). Such a pressure effect, which has never been reported as a significant operating parameter, can be assigned to higher dissolved CO_2 or H_2 in the liquid. Both biogases could interfere with the metabolic pathways, CO_2 is also a buffering agent (considering the respective carbonates) and, therefore, possibly exerts a synergetic effect on maintaining the pH level closer to the optimal neutral conditions.

Differing from what was reported elsewhere,[32] no mildly acidic conditions were necessary for avoiding methanogenesis (ESI, Fig. S2A–C†), even at pH 7 and 55 °C (ESI, Fig. S1D–F†). It turned out that high carboxylate concentration is sufficient for avoiding methanogenesis, all the more so when continuous fermentations rather than batch culture systems would reasonably be conducted for MCC production in industrial environments (*i.e.* $D_{Acid} > D_{met}$, thus methane producers washout).

The maximum C6 instant productivity detected under the optimal conditions (6.2 g L^{-1} d^{-1}) was notably higher than the one reported for the fermentation of corn beer in the semi batch mode (2.1 g L^{-1} d^{-1})[16] or continuous mode (3.38 g L^{-1} d^{-1}).[32] Furthermore, it is also higher than that reported for the chain elongation with wine lees (1.77 g L^{-1} d^{-1}).[20] Besides, the obtained ethanol to C6 yield is 1.00 g g^{-1}, which is similar to those reported when feeding a pure culture of *C. kluyveri* with commercial ethanol and acetic acid or when feeding a mixed culture with corn beer (1.11 g g^{-1}).[13,29] The obtainment of such high values was assigned to other organic compounds occurring in the agro-wastes (mainly sugars in the case of GP_{Fresh}). Thus, an overall fresh weight yield of 64 kg of C6 per ton of GP_{Fresh} could be produced which is comparable to the best reported production from actual agro-industrial by-products without ethanol addition (*e.g.* corn beer and wine lees).[13]

Production of highly concentrated hexanoic acid. Anaerobic fermentation of GP_{Fresh} was upscaled to 20 L (16 L working volume) by applying the most performing experimental conditions observed in the previous screening test (pH 7, 37 °C and $P > P_{atm}$). This yielded 22 g L^{-1} at day 13 (see ESI Fig. S6†). The GP^{Acid}_{Fresh} solid fraction was separated and stocked (for studying its biomethane potential). The addition of HCl (0.31 M final concentration) to 8.5 L of broth allowed reducing the pH level to 2.2 and insolubilizing 55% of the produced C6. The immiscible recovered C6 (100 g) showed a purity of 87% as determined by HPLC, COD and GC-MS analyses (see ESI Fig. S8 and S9†). The main impurities were butyric and octanoic acid, whereas traces of acetic acid and esters of the C_4–C_8 carboxylic acids were detected in very low concentrations. Indeed, the presence of GP_{Fresh} derived impurities was expected as indicated by the reddish colour of the recovered

product, whereas commercial C6 is transparent. Regarding the water phase still including the soluble C6 fraction (~10 g L^{-1}), it could be potentially recycled or mixed with GP_{Fresh} in the second anaerobic fermenter for the production of caprylic acid (C8). In fact, *n*-caprylate was also produced: *ca.* 0.4 g L^{-1} (see ESI Fig. S16†).

The proposed fermentation at pH 7 (with high titer, productivity and easy downstream) could represent a less energy dependent process in comparison with the previously reported C6 production strategies (pH 5.5 with in-line pertraction C6 recovery) where productivity depends on the recycling pumping rate.[32] Although HCl was used in the present work to demonstrate the ease of C6 separation, a greener process could be considered when applying a bipolar membrane electrodialysis step (or electro-electrodialysis) that could separate Na^+ (to be recycled to the fermentation step) while insolubilizing C6 that could be recovered.[33]

The reported achievements encourage an optimistic outlook for a techno-economic feasible GP valorisation without subsidy requirement. In Italy, for instance, more than 25 ktons of C6 per year could be obtained just from red GP_{Fresh} (considering 55% recovery of total produced C6). This represents a potential equivalent income of 78 M€ per year, which is 1.9 times higher than that for ethanol recovery. Importantly, similar results are being obtained using GP arising from the white wine production (data not shown), this shows the possibility of exploiting also that type of GP through the same valorisation route. Regarding revenues, Table 1 shows that the cost estimated for the recovery of ethanol by distillation is higher than the market price, whereas its upgrade into C6 results at a slightly competitive price. The considered OPEX production costs were (i) GP truck transport; (ii) C6 production by fermentation (up-stream); (iii) ethanol distillation (down-stream cost estimated according to the method previously reported elsewhere[19]) and (iv) C6 down-stream cost for 55% C6 recovery (estimated at 20% of total cost in accordance with the simplicity of the procedure). Remarkably, the ethanol recovery by distillation is hardly economically feasible when considering its averaged international market price, *i.e.* the added value would range between −30 and 5 EUR per ton of GP_{Fresh}. Differently, when considering the ethanol subsidised price (1338 EUR per ton),[34] the added value would be 17 EUR per ton of GP_{Fresh}. On the other hand, C6 valorisation route with 55% recovery yield (Table 1) allows achieving, without subsidies, 17 EUR per ton of GP_{Fresh}. The added value would be 46 EUR per ton of GP_{Fresh} when considering, as discussed above, a C6 recovery yield of 70%. Interestingly, the highest expense shown in Table 1 for both final products is that of GP transport, which represents 92% and 64% of the ethanol and C6 total costs, respectively. The economy of the energy intensive ethanol distillation is strongly based on the scale, *i.e.* requiring GP transport (costs and CO_2 emissions) to large scale central plants. Diversely, the simple MCC production can be carried out at the winery, using simple technologies with which they are already familiarised (anaerobic fermenter and solid–liquid and liquid–liquid separators). Avoiding transportation represents another economic and environmental advantage for the proposed valorisation route, allowing us to achieve 55 or 83 EUR per ton of GP_{Fresh} for a C6 recovery yield of 55 or 70%, respectively, and already considering the product transport to a client (30 km) and the GP^{Acid}_{Fresh} transport to a local (10 km) AD plant (this further valorisation assessment is shown in the following section).

Table 1 Cost estimation for ethanol recovery (EtOH) and concentrated hexanoic acid production (C6)

	Costs (EUR per ton of product)				
Product	GP_{Fresh} transport [a]	Up-stream	Down-stream	Total	Market price (€ per ton)
EtOH	934	0	80[c]	1014	852[d]
C6	1699	566[b]	400	2665	3000[e]

[a] 30 km distance at 2€ per ton of GP_{Fresh} required. [b] Fermentation cost (20€ per ton of GP_{Fresh} required) based on previously reported data.[35] [c] Distillation cost based on previous reported data,[19] namely: $\left(\frac{75\,000\text{BTU Nat gas}}{\text{Gal EtOH}} \times 0.6 \times \frac{5.7\times10^{-6}\text{EUR}}{\text{BTU Nat gas}}\right) + \left(\frac{0.925\text{kWH}}{\text{Gal EtOH}} \times 0.05 \times \frac{0.08\text{EUR}}{\text{kWH}}\right)$. [d] Considering the average of 1.6–3.2 € per Gal. [e] Reported previously elsewhere.[36]

Although GP_{Fresh} valorisation through the production of C6 is sustainable in itself, the added value associated with GP-derived products (EUR per ton of GP_{Fresh}) could be further increased by exploiting the GP seed fraction for extracting oils as previously revised elsewhere.[37] Although such a step was not assessed in this work, it could be easily integrated since the seeds do not represent a substrate for the chain elongation fermentation and thus they could be separated thereafter. Instead, the work did focus on the industrial interest to evaluate the technical feasibility of directly transforming the separated C6 (as obtained) into other products, *e.g.* solvents, biofuels or biopolymers. Hence, two innovative proof-of-concept and a complementary valorisation step are presented in the following sections.

Production of hexanoic acid derived ester and alcohol

Catalytic heterogeneous hydrogenation was carried out on the as-obtained C6. The commercial monometallic Re/C catalyst was adopted since it shows high resistance to poisoning and it is much more economic than other metallic catalysts (*e.g.* Pt, Pd, Ru) generally used in the hydrogenation of carboxylic acids.[38] The applied reaction conditions allowed conversion of 94.7% of the hexanoic acid (Fig. 3A) after 9 h. The two leading products were hexyl hexanoate and 1-hexanol (Fig. 3B) with an overall yield of *ca.* 75%, whereas the main by-products derived from hexanoic acid (with yields < 1%) were n-hexane, hexyl-butanoate and hexyl octanoate (ESI, Fig. S17†). These last to be derived from butyric and octanoic acids present in crude C6. Moreover, butanol as well as octanol and their mixed esters derived from those acids, were also ascertained. Recycling tests were performed in order to evaluate catalyst stability in the presence of this crude starting material. Briefly, the catalyst was recovered from the reaction mixture by filtration and re-used within two subsequent recycling runs performed under

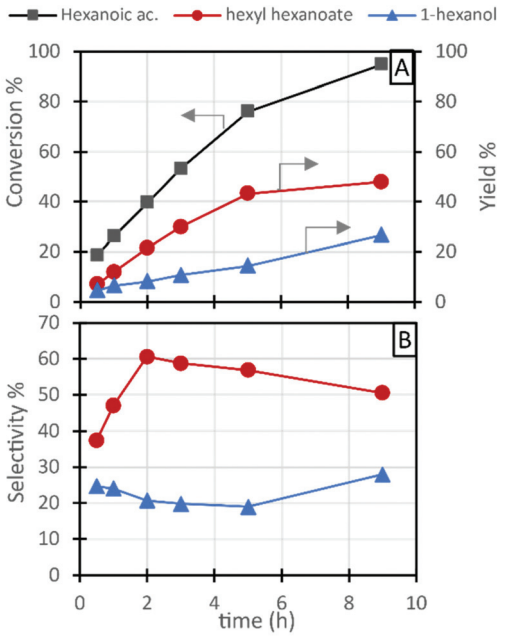

Fig. 3 Catalytic hydrogenation performances along the reaction time, namely: (A) GP-derived C6 conversion as well as the respective hexyl-hexanoate and 1-hexanol yields and (B) selectivity.

the same reaction conditions. During these three cycles the catalytic activity and selectivity remained unchanged, evidencing the stability of the adopted rhenium catalyst.

Interestingly, when using pure hexanoic acid under analogous reaction conditions, 95% conversion was achieved after 6 h of reaction time with an analogous profile of the two reaction products. Such better performance was assigned to the absence of other acids, traces of esters and water which had been ascertained in crude C6 used as obtained in the previously reported separation step.

It is worth noting that the as-obtained mixture of 1-hexanol and hexyl hexanoate can represent an extremely promising blendstock for both diesel and bio-diesel fuels. In fact the addition of each of these components resulted in good diesel engine performances, is also reported to have reduced the HC, CO and soot emissions.[39,40] The use of the mixture 1-hexanol/hexyl hexanoate, up to now never tested without separation of the components, can represent a novel cheap biofuel.

Production of hexanoate-derived mcl-PHAs

Fermentation. The obtained highly pure C6 was also of interest from the perspective of use for bio-elastomer production: notably mcl-PHAs rich in 3-hydroxyhexanoate monomer (3-HHx). Not only is it true for the obtainment of a higher added value product, but also because the highly concentrated C6 would make proper fed-batch culture system feasible (avoiding high dilution, crucial for the process economy).[41]

Hence, a dual-phase process was set-up to test such production in a bench-top bioreactor. Briefly, this fermentation strategy allows employing C6 specifically on the biopolymer accumulation (under N-limiting conditions) inside cells that were first grown under balanced conditions using glucose as the carbon source.

Fig. 4A shows the obtained results. The growth phase lasted 8.5–9 hours (μ = 0.42 h^{-1}), when the cell dry weight (CDW) and PHA concentration were 7.13 ± 0.35 g L^{-1} and 0.77 ± 0.01 g L^{-1}, respectively. Thereon, the accumulation phase was triggered by feeding the GP derived C6 or commercial C6 (the latter serving as control conditions). To avoid C6 accumulation in the culture broth, a pH-stat feeding strategy was applied. Such automatism resulted in feeding rates of 0.87 mL h^{-1} (from 9 to 32 h) and 0.40 mL h^{-1} (from 32 to 50 h). The specific accumulation rates ranged between 0.035 and 0.010 h^{-1}, whereas C6 concentrations were negligible throughout the accumulation phase. No difference in the fermentation performance was observed between GP derived-C6 and commercial C6 fed cultures: CDW and PHA yields were identical (Fig. 4B). Such yields are lower than those previously reported (0.37–0.69 g g^{-1}) when octanoic or nonanoic acids were fed to the same *P. putida* strain.[42,43] However, as discussed by Le

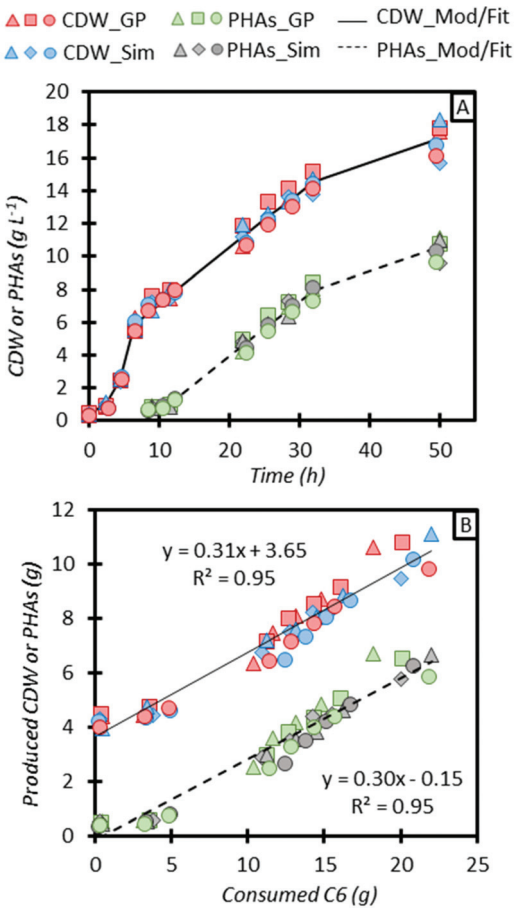

Fig. 4 PHAs production from GP derived (GP) or commercial C6 (Sim). Graph A shows the experimental and modelled (Mod) concentrations of CDW and PHAs. Graph B shows the CDW and PHA production yields with respect to C6 consumed.

Meur *et al.*,[42] this was assigned to the partial utilization of C6 by the cells to conclude the growth phase. Indeed, considering in Fig. 4B only the points for C6 consumption above 10 g, the fitting slope would indicate a yield of 0.35 g g^{-1}. Further optimisation is required (it is beyond the aim of this work), nonetheless the obtained outcomes demonstrate the convenience and reliability of the proposed integrated strategy: the GP-derived C6 supplemented to feed a *P. putida* culture led to yield of 17.04 ± 1.03 g L^{-1} of cells. Both, the 61 ± 1% of PHA content (g_{PHAs} g_{CDW}^{-1}) and the 50 h of total fermentation time were in the mean performances for achieving economically feasible production.

Polymer characterisation. NMR analyses allowed us to determine that the biopolymer was mainly composed of 3-HHx (90%), the others being 3-hydroxyoctanoate (7%) and 3-hydroxydecanoate (3%; see the ESI: Fig. S23 and S24†). This is the first time a mcl-PHA with such a high content on 3-hydroxyhexanoate was produced by employing an agro-industrial derived MCC. GPC analysis indicated a molecular weight of 145–133 kDa and a polydispersity of 1.9. Moreover, DSC analysis showed a T_g of −31 °C and no T_m was detected (ESI, Fig. S25†), whereas TGA analyses show a T_d of 287 °C (ESI, Fig. S26†). This is in accordance with the obtainment of a biopolymer with a high content of 3-HHx (an amorphous polymer).[44,45] Such a sticky and transparent biopolymer (Fig. 5) is a promising product for toughening brittle polymers (PLA or PHB for instance) or making them more flexible, which might be employed for adhesives or biomedical applications (*e.g.* drug delivery). However, its production needs upscaling for obtaining more polymer per batch which allows obtaining a thorough characterisation and assessing possible final applications at the minimal scale.

Biogas production from GP^{Acid}_{Fresh} solid fraction

A biomethane potential study was conducted to verify if the GP^{Acid}_{Fresh} solid fraction (containing 30% TS and 91% VS) could be sent to an anaerobic digestor to valorise the leftover material. The results shown in Fig. 6 show that GP^{Acid}_{Fresh} allowed producing almost twice (192 NmL gSV^{-1}) the amount of methane previously reported for GP^{Acid}_{Deph} (127 NmL gSV^{-1}).[9] Such a difference between both pre-fermented GPs was assigned to the low selectivity that supercritical fluid extraction had toward polyphenols, *i.e.* the first step of the previous valorisation scheme (Fig. 1) solubilizes other digestible compounds alongside polyphenols. Furthermore, both yields fit the range previously reported elsewhere for different GPs without pre-treatment (128–375 $mLCH_4$ gVS^{-1}).[46] Bearing in mind (i) the MCC production conditions (14.8% TS) and the solid–liquid separation after it (see above), (ii) the TS and VS contents in the resulting GP^{Acid}_{Fresh} solid fraction and (iii) the experimental methane yield, such a final valorisation step would allow producing 75 N m^3 of methane per ton of GP_{Fresh}. Considering the volumetric heat power of methane (10.47 kW h m^{-3}), the heat to electricity conversion yield (*ca.* 40%), the electricity price for non-household consumers (0.08 EUR kW h^{-1})[47] and the AD (with electricity cogeneration) cost (0.06 EUR kW h^{-1}),[48] would render a potential extra income of 5 EUR per ton of GP_{Fresh}. In this line, although it was not assessed in this work, the carboxylic acids remaining in the aqueous phase after C6 separation could also be valorised by such AD processes.

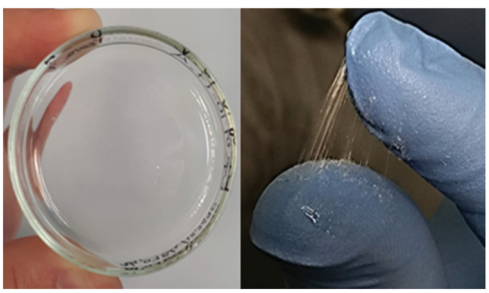

Fig. 5 Images showing a 4 mm layer of biopolymer plated on a Petri dish and its stickiness behaviour of the GP derived mcl-PHAs.

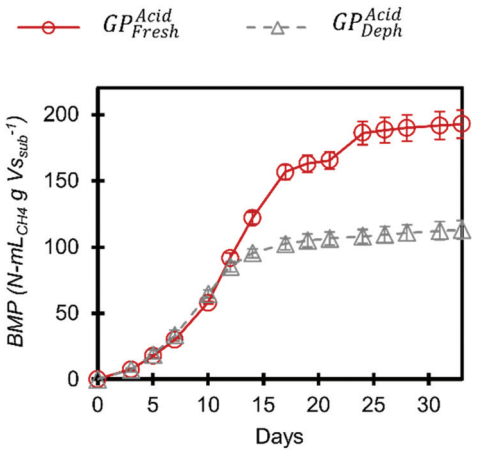

Fig. 6 Biomethane potential of the pre-treated GP: dephenolised and fermented ($GPGP^{Acid}_{Deph}$) or only fermented (GP^{Acid}_{Fresh}).

Experimental

Chemicals and grape pomace

The standard mixture of carboxylic acids, salts for the mineral medium, hexanoic acid and glucose were purchased from Merck.

The experimental GP_{Fresh} from red grape varieties was kindly provided by Caviro Distillerie (Faenza, Italy), arising directly from red wine production without any pre-treatment It contained 56.83 ± 0.05% humidity and 93.28 ± 0.07% volatile solids (VS), determined as reported elsewhere.[9] It was stocked at 4 °C until use.

Production of hexanoate from GP_{Fresh}

Screening the best temperature, pH and pressure conditions. The acidogenic inoculum was the same as that

employed earlier to ferment GP_{Deph}.[9] The feasibility of producing MCCs from GP_{Fresh} was tested at different pH levels (5, 6 and 7) and temperatures (37 and 55 °C). To this aim, the study was carried out in 100 mL Pyrex bottles (47 mL of working volume) tightly closed with a modified Pyrex screwcap allowing gas sampling. In contrast, just the optimal conditions (pH 7 and 37 °C) were used to study the operating pressure effect on the GP_{Fresh} fermentation. In this case a balloon was installed in the Pyrex bottle for obtaining a dynamic headspace volume which allowed maintaining the pressure close to the atmospheric pressure. The inoculum to substrate ratio (18.0 ± 0.7 mg_{VS} of inoculum per g_{VS} of the substrate) and the total solid (TS) content (14.82 ± 0.47%) were identical under all the experimental conditions. Experiments under each experimental condition were carried out in triplicate and a control condition was set up by filling microcosms only with water and the inoculum. The last one contained a negligible amount of total VFAs (0.5 ± 0.1 g L^{-1}) from the previous experiment. Microcosms were incubated on an orbital shaker (150 rpm). These microreactors were periodically monitored for biogas and organic acid productions, as well as pH control, in agreement with the same sampling and analytical procedures previously reported.[9]

Production of high quantities of hexanoic acid. Further anaerobic fermentation was carried out under the optimal conditions previously determined (pH 7, 37 °C and under pressure) using a 20 L plastic drum (12 L working volume) supplied with a modified screwcap with a silicone septa. The drum was shaken on an orbital shaker (150 rpm). As in previous microcosm tests, the fermentation was periodically monitored for biogas as well as organic acid productions and pH control. After the completion of fermentation, the solid fraction of GP^{Acid}_{Fresh} was separated by filtration (porous diameter 1 mm). The permeate fraction was centrifuged (5000 rpm for 5 min) to collect the biomass. Then, HCl (37%) was added to the GP^{Acid}_{Fresh} liquid fraction to bring pH down to 2.2. Next, the liquid was slowly transferred into a second bottle (gently mixed with a magnetic stirrer) where the two liquid phases were separated; the upper phase was hexanoic acid that was separated by overflow (see ESI Fig. S7†). Finally, the hexanoic acid rich stream was centrifuged (5000 rpm for 5 min) to separate suspended solids precipitated under HCl induced acidic conditions. The hexanoic acid concentration was determined in both separated streams in order to evaluate recovery yield and purity.

Production of hexanoate-derived ester and alcohol

C6 hydrogenation was performed in an electrically heated 300 mL Parr autoclave equipped with a P.I.D. controller (4848). The catalyst (commercial Re/C 5 wt%) was loaded adopting a substrate/catalyst ratio of 270 mol mol^{-1}, determined considering the employed substrate as pure hexanoic acid. The catalyst was introduced in the autoclave and pre-reduced adopting a wet pre-reduction in methanol (temperature 180 °C, hydrogen pressure 90 bar, time 6 h). Hence methanol was removed from the reaction system under vacuum and the crude substrate was introduced by suction. The reactor was pressurized with hydrogen up to a value of 115 bar determined at a reaction temperature of 180 °C, pressure was held constant by automatically feeding more hydrogen. The reaction mixture was stirred using a mechanical overhead stirrer. At the end of the reaction, the reactor was rapidly cooled at room temperature by blowing air, the autoclave was depressurized and the reaction mixture was filtered to remove the catalyst and finally analysed through GC/FID chromatography and GC/MS spectroscopy. Hexanoic acid conversion was calculated as follows:

$$\text{Conversion}(\text{mol}\%) = \frac{\text{mol of hexanoic acid converted}}{\text{mol of hexanoic acid in the starting mix}} \times 100$$

Whilst selectivity and yields were calculated according to the following equations:

$$\text{Selectivity of } i(\text{mol}\%) = \frac{\text{mol of product } i \times \text{num. of } C_6 \text{ groups}}{\sum \text{mol of product } n \times \text{num. of } C_6 \text{ groups}} \times 100$$

$$\text{Yield of } i \text{ (mol\%)} = \text{Selectivity of } i \text{ (mol\%)} \times \text{conversion (mol\%)}$$

Production of hexanoate-derived mcl-PHAs

Fermentation. The experiment was performed according to a dual-phase process, in twin bench-top bioreactors (MiniFors2, 2L). To this end, LB pre-cultured *Pseudomonas putida* (DSMZ 6125) was harvested by centrifugation (10 000 rpm for 8 min at 10 °C), resuspended in 30 mL of the culture medium and inoculated (to an initial absorbance at 600 nm of *ca.* 1 A.U.) in 570 mL of a mineral medium previously described elsewhere.[49] It was slightly modified in order to better balance the growth and accumulation phases: 3.5 g L^{-1} of $(NH_4)_2SO_4$ instead of 4.7 g L^{-1}. First cells were grown in the batch mode under balanced nutrient conditions, providing glucose (7.5 g L^{-1}) as a carbon/energy source. When glucose was almost depleted (0.1 g L^{-1}), the second phase (fed-batch culture system) was set-on by feeding the C6 under a pH-stat mode, hence avoiding cell inhibition from organic acid.[41] Aeration and stirring were automatically controlled in cascade in order to maintain $pO_2 > 40\%$.

The effects of impurities present in the C6 derived from GP_{Fresh} were evaluated using parallel experimental conditions in which the grown cells were fed with commercial hexanoic acid (during the second phase).

Both conditions were applied three times. Fermentations were monitored by periodically sampling 5 mL of the fermentation broth for determining the concentration of glucose, C6, NH_4^+, PO_4^{X-}, CDW and PHAs (calculated from PHAs%, in $g_{PHAs}\ g_{CDW}^{-1}$), as previously reported elsewhere.[41]

At the end of each fermentation, cells containing PHAs were harvested by centrifugation (10 000 rpm for 8 min at 10 °C). After a washing step in physiological solution, the cells

were dried in an oven at 60 °C and stocked for PHA extraction and characterisation.

Biogas production. A BMP study was carried out for the solid fraction of GP^{Acid}_{Fresh} with the same experimental set-up and procedures as previously described for $GP^{Acid}_{Deph;Solid}$.[9]

Analytical procedures

Nutrient and metabolite concentration. Glucose, PO_4^{x-}, carboxylic acids and ethanol titers were determined by HPLC-RID, using the same procedures reported previosuly.[9] An ion selective electrode was used to determine NH_4^+ concentration.[50]

Highly concentrated GP-derived C6 characterisation. The purity was determined by preparing three samples at different dilutions and by comparing the HPLC-RID results with those obtained from the other three samples prepared at the same dilution ratios but using commercial C6. Besides, C6 purity was determined by analysing the COD of the mentioned six samples (using VARIO colorimetric vials[9]) and comparing in a second parity plot (see ESI Fig. S5†).

PHA extraction and characterisation. PHAs were recovered and purified using chloroform and ethanol. Thereafter, a set of analyses were carried out for the biopolymer characterisation, namely: (1) NMR-C and NMR-H for monomer composition, (2) differential scanning calorimetry (DSC) for determining T_m and T_g, (3) gel permeation chromatography (GPC) for M_w and PDI and (4) thermal gravimetric analysis (TGA) for T_d. All related procedures were previously reported elsewhere[26,44]

PHA content inside the cells. It was determined by GC-FID analysis after the application of the methanolysis procedure on the dried samples.[51] In this work, the standard used was the actual biopolymer produced, purified and characterised in this work as mentioned above.[44]

Biogas production and composition. Produced biogas was measured using a glass syringe and its composition was determined using an Agilent 3000A μGC as previously described elsewhere.[9]

Total and volatile solids were determined by the conventional gravimetric method.[9]

Conclusions

A novel multipurpose approach for the valorisation of red grape pomace (GP) was assessed. It allowed improving the valorisation of the contained ethanol through the production of medium chain carboxylates (MCCs). Indeed, differently from previous reports, the neutral pH level allowed us to obtain high concentrations of hexanoic acid (C6) by anaerobically fermenting the red GP. Besides, for the first time, the pressure was reported as a significant operating parameter for achieving high MCC production. In turn, the obtained high titer permitted easy recovery of the C6 with 87% purity. A preliminary cost evaluation indicated that such a strategy resulted competitive with C6 market prices, *i.e.* this novel route could represent a potential option for a truly sustainable GP management as it would not need subsidies. An additional advance in the prospective application of cascading biorefinery, lies in the highly concentrated C6 which was assessed for the first time as a reagent and as a fermentation substrate. The catalytic hydrogenation of crude hexanoic acid allowed producing a hexyl-hexanoate/hexanol mixture which represents a promising blendstock for fuels. Alternatively, medium chain length polyhydroxyalkanoates (mcl-PHAs) were produced with *P. putida*. The biopolymer was mainly composed of 3-hydroxyhexanoate (90%), completely amorphous and sticky. Complementarily, a BMP study indicated that the exhausted solid could be used for producing biomethane. To the very best of our knowledge this is the first time that chemicals and mcl-PHAs are produced using actual highly concentrated MCCs derived from agro-industrial by-products.

Author contributions

Gonzalo A. Martinez: conceptualization, investigation, formal analysis, supervision, visualization, writing – original draft. Salvatore Puccio, Joana M. B. Domingos and Elena Morselli: investigation. Claudio Gioia, Paola Marchese and Annamaria Celli: resources. Anna Maria Raspolli Galletti: conceptualization, formal analysis, supervision, visualization, writing – review & editing. Fabio Fava: supervision. Lorenzo Bertin: conceptualization, supervision, funding acquisition, project administration, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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