Chemical and Biological Technologies in Agriculture

Polyhydroxyalkanoates (PHAs) from dairy wastewater effluent: bacterial accumulation, structural characterization and physical properties

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Abstract

Background: To establish bioplastics as a real alternative to conventional plastics, high production costs must be constrained by using different kinds of wastewater streams as organic substrates and novel microbial strains as material-accumulating bacteria with high performance. Volatile fatty acids (VFAs) from the effluent of dairy wastewater biodigestion represent a new and inexpensive feedstock, which was used in this study for biopolymer production through microbial processes.

Results: *Cupriavidus necator* DSM 13513 was particularly able to accumulate PHAs when operating in fed-batch mode by limiting the oxygen level together with intermittent feeding of a carbon source; maximum poly-β-hydroxybutyrate (PHB) accumulation was achieved in 48 h without compromising microbial growth. The complex VFAs mixture from the digestate did not influence PHA homopolymer accumulation. In fact, structural characterization by NMR analysis revealed PHB synthesis by *C. necator* DSM 13513 grown with different VFAs mixtures. Moreover, the bioplastic disk obtained from *C. necator* DSM 13513 cells grown on VFAs from digested dairy wastewater effluent presented good thermic properties and low affinity to water.

Conclusions: Overall, the results make digested dairy wastewater effluent suitable for PHB production for specific biobased industrial applications.

Keywords: Biodigestate, Volatile fatty acids, *Cupriavidus necator*, Poly-β-hydroxybutyrate, Biopolymer properties

Background

One of the key challenges of this century in the environmental field is to progressively replace plastics derived from fossil fuels with biobased, biodegradable and compostable plastics derived from nonfood renewable sources [1]. The European Commission is promoting the "Circular Economy Action Plan" to replace conventional plastics generally utilized for single-use products with bioplastics by 2021 (EUR-Lex-52018PC0340). To establish bioplastics as a real alternative to conventional plastics, high production costs have to be constrained by using different kinds of wastewater streams as organic substrates and novel microbial strains as material-accumulating bacteria with high performance. This aim could be achieved by eco-designing sustainable biopolymer production methods based on microbial biobased processes [2, 3]. Among biopolymers, microbial polyesters known as polyhydroxyalkanoates (PHAs) are biodegradable plastics synthesized by different bacteria from a

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range of substrates, including sugars and fatty acids [4]. Volatile fatty acids (VFAs), such as acetic, propionic, and butyric acids, are potentially renewable carbon sources [5] that could be used for biogas production [6], electricity generation [7] and PHA synthesis [8]. Therefore, effluent from dark fermentation H_2 processes, containing large quantities of VFAs, represents an interesting feedstock for the PHA production process and an opportunity to enhance the treatment of such effluents [9]. In this context, in recent decades, many studies have focused on energy sources, such as hydrogen produced from dairy wastes [6, 10], showing options to valorize this effluent in the chemicals industry or by other biological systems for energy recovery (e.g., methane). In fact, an interesting integrated system can be designed to combine an energy source (mainly hydrogen) and biopolymer production (PHA) utilizing an acid-rich wastewater stream [4]. For these reasons, effluent from the acidogenic digestion of dairy wastes is also an inexpensive acid-rich wastewater stream for PHA production [11]. In particular, whey and buttermilk are suitable by-products that can be submitted to the hydrolysis–acidification step under anaerobic conditions to produce hydrogen and VFAs. In fact, this wastewater stream mainly includes VFAs, lactic acid, alcohols and residues that are unhydrolyzed [9]. The resulting VFAs with lower carbon numbers are the main precursors for PHA production by many microbial species.

One well-known PHA-accumulating microbial species is *Cupriavidus* (*C.*) *necator*, also known as *Wautersia eutropha* and *Alcaligenes eutrophus*, formerly classified as *Ralstonia eutropha* [12]. Gram-negative bacterial strains belonging to this species are able to accumulate higher yields of PHAs from VFAs as intracellular carbon and energy reserve granules, depending on the strain and operating mode [13]. Indeed, PHAs and their copolymers are classified based on the length of the alkyl side chain present in the PHA, which is correlated to the substrate specificity of PHA synthases. Typically, *C. necator* produces short-chain (C3–C5) hydroxyalkanoic acids (PHA$_{SCL}$), which have alkyl side chains such as P(3HB), P(3HV), P(4HB) and the copolymer P(3HB–co–3HV) [14]. Therefore, it is essential to determine the chemical composition of these compounds to evaluate their potential for industrial use. Gas chromatography/mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR) are among the most widely used analytical methods for the chemical characterization of PHA structures [15] since their coupled use allows a careful and unambiguous molecular description to be obtained.

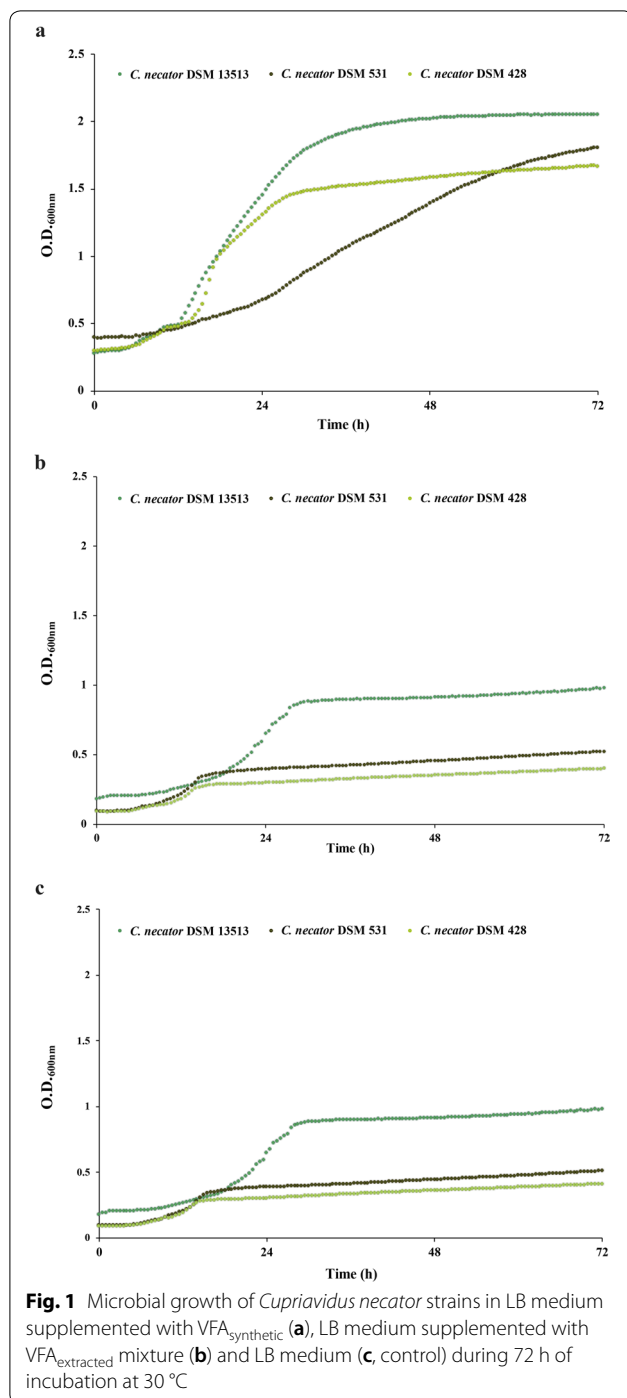PHAs represent a heterogeneous family of biobased (co)polyesters constituted by more than 150 different monomers, resulting in materials with extremely different properties that are not always fully competitive compared to conventional thermoplastics [16]. Although PHAs can show poor physical properties due to secondary crystallization and a slow nucleation rate, they have physical properties comparable to those of petroleum-based thermoplastics. In particular, poly-β-hydroxybutyrate (PHB) exhibits remarkable physical properties comparable to those of polypropylene (PP) and polyethylene (PE). The stereochemical regularity of its structure leads to a highly crystallized homopolymer (crystallinity up to 70%), contributing to its excellent physical properties [17]. Kinetic data obtained from thermogravimetric (TGA) measurements are very useful for understanding thermal degradation processes. Many studies have revealed that the degradation occurs rapidly near the melting point mainly through a random chain scission process based on the typical structures of pyrolysis products, i.e., crotonic acid and oligomers with a crotonate end group [18, 19]. On the other hand, studies have also shown that the parameters of melt processing must be optimized to avoid or restrict this phenomenon, which reduces the processing window.

Hence, the PHA production chain through biological processes involves bacterial strains with high biotechnological performance, low-cost feedstock selection, fermentation technology and downstream technologies [20]. The present study analyzed many aspects of the sustainable production of biobased plastic films composed of PHAs using organic acids obtained from the first step of biodigestion of dairy wastewater. Different strains belonging to *C. necator* were assayed to study their ability to accumulate PHAs while growing on organic acids from dairy wastewater biodigestion effluent as well as a synthetic mixture of pure acids as growth media. Their biotechnological performances were compared by detecting and monitoring cell growth, PHA formation and PHA quantity. In addition, the PHA monomeric composition was qualitatively analyzed by GC/MS and structurally characterized by NMR. The thermal degradation behaviors of PHA samples were characterized under isothermal conditions. Moreover, to understand the interaction of PHA with water, the water absorption kinetics were studied at 40 °C.

Results and discussion
Ability of *C. necator* strains to grow in media containing VFAs
A preliminary screening of the strains *C. necator* DSM 13513, DSM 531 and DSM 428 was performed on the basis of their capacity to grow in the presence of a mixture of VFAs. Figure 1 shows the growth curves of the three strains in Luria–Bertani (LB) medium with VFA$_{synthetic}$ (Fig. 1a), in LB medium with VFA$_{extracted}$

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Fig. 1 Microbial growth of *Cupriavidus necator* strains in LB medium supplemented with VFA$_{synthetic}$ (**a**), LB medium supplemented with VFA$_{extracted}$ mixture (**b**) and LB medium (**c**, control) during 72 h of incubation at 30 °C

Fig. 2 PHB production (%) and microbial counts (log CFU mL^{-1}) of *C. necator* DSM 13513 in batch cultures grown in LB medium supplemented with VFA$_{synthetic}$ (**a**), the VFA$_{extracted}$ mixture (**b**) and LB medium (**c**, control) for 48 h of incubation at 30 °C

(Fig. 1b) and in LB medium (control; Fig. 1c). All *C. necator* strains showed enhanced growth from the excess carbon source due to the added organic acid (VFA$_{synthetic}$) in the culture medium. In particular, *C. necator* DSM 13513 showed the highest growth, achieving 1.97 O.D.$_{600\ nm}$ at 40 h in the presence of VFA$_{synthetic}$ (Fig. 1a), in contrast to the control assay (0.90 O.D.$_{600\ nm}$; Fig. 1c). These
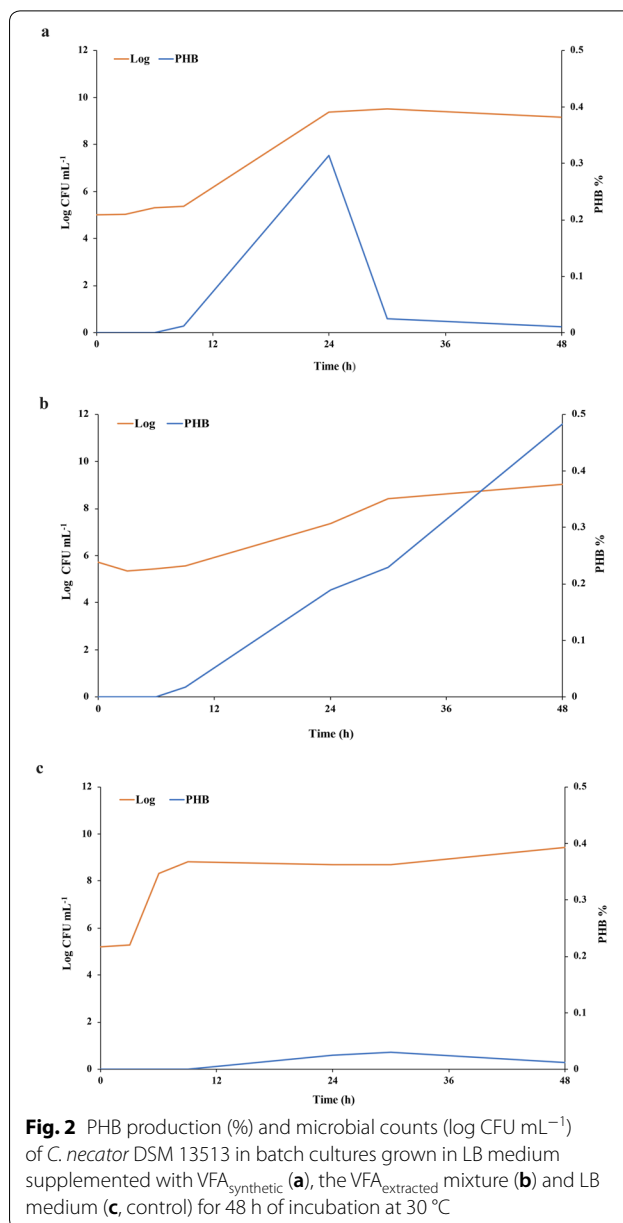
results demonstrated that these bacterial strains were well adapted to the acidogenic nutritional conditions due to the addition of the VFAs mixture. The strain *C. necator* DSM 13513 showed the best growth (0.97 O.D.$_{600\ nm}$; Fig. 2b) in the presence of VFA$_{extracted}$ with a trend similar to that detected in LB liquid medium (control tests without VFAs; Fig. 1c). Based on the chemical characterization, the main difference between VFA$_{synthetic}$ and VFA$_{extracted}$ mixtures was the presence of ethanol in the latter (20.24 ± 0.52 g L^{-1} ethanol). Ethanol concentration in the medium with VFA$_{extracted}$ mixture did not affect the growth of the bacterial strains, as shown by comparison

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with the control test without VFAs (Fig. 1b, c). In fact, according to Obruca et al. [21], the exposure of *C. necator* to ethanol can enhance PHB production without inhibiting microbial growth. Indeed, at low concentrations, organic acid mixtures can be effectively utilized by *C. necator* as substrates for bacterial growth, although organic acid mixtures could be toxic to cells [22].

This preliminary screening supported the selection of the strain *C. necator* DSM 13513 for further investigations on PHA production from VFAs.

Batch culture experiments in media containing VFAs

On the basis of the previous results, batch culture experiments (600 mL) with *C. necator* DSM 13513 and media containing $VFA_{synthetic}$ or $VFA_{extracted}$ were conducted to evaluate PHA synthesis. After 24 h of incubation at 30 °C at the up log phase of growth, the PHB content reached 0.31% (Fig. 2a) in the medium containing $VFA_{synthetic}$, 12-fold higher than that accumulated in the test control (optimal medium without $VFA_{synthetic}$) at the same time (0.025%) (Fig. 2c). In fact, various bacteria are able to utilize acetic, propionic and formic acid as substrates for PHA synthesis [23]. Among them, *C. necator* is known to be one of the best PHB-accumulating bacteria [24], even if the biotechnological performance is strain dependent. Similar performance was also demonstrated in this research since *C. necator* DSM 13513 was selected as the best strain to accumulate PHB from the fatty acid-containing effluent of an anaerobic process fed with dairy wastewater. After 24 h of growth, the cellular concentration of *C. necator* DSM 13513 was higher in the batch cultures with $VFA_{synthetic}$ (9.38 log CFU mL^{-1}; Fig. 2a) than in the control medium (8.70 log CFU mL^{-1}; Fig. 2c). $VFA_{synthetic}$ addition prolonged the exponential growth phase by delaying the start of the stationary phase, where a decrease

in the percentage of PHB was observed. This result was probably due to the consumption of PHB accumulated in the cells as an energy reserve due to the lack of a carbon source. Moreover, several microbial strains show PHB accumulation until the stationary phase, as reported by Bhatia et al. [25], who studied the recombinant *Escherichia coli* strain SKB99.

In the presence of $VFA_{extracted}$, the strain *C. necator* DSM 13513 showed a particular trend in prolonging the log phase after 24 h of incubation, with a microbial count ranging from 7.37 log CFU mL^{-1} at 24 h to 9.03 log CFU mL^{-1} at 48 h (Fig. 2b). This behavior was related to an increase in PHB production; 0.2% PHB was achieved after 24 h of incubation, with a significant increase in PHB until reaching 0.48% at 48 h (Fig. 2b). Even though *C. necator* DSM 13513 showed a slower growth rate, PHB synthesis was stimulated by the presence of $VFA_{extracted}$ in the medium. Interestingly, the trend of the PHB curve did not reach a plateau after 48 h, demonstrating the opportunity to extend the accumulation time in the cells. However, at 48 h, the same microbial concentration was observed in all experimental conditions.

As previously described, the presence of ethanol in $VFA_{extracted}$ did not influence microbial growth, but Obruca et al. [21] demonstrated that ethanol could increase the PHB yield by approximately 30%. Ethanol is considered a precursor of PHA, particularly HV [26] or HB [27], but there is no clear indication of the microbial pathway.

PHA accumulation in the media with and without VFA mixtures was observed under a fluorescence microscope, as shown in Fig. 3. An increase in fluorescence was observed after 24 or 48 h of incubation, corresponding to the maximum PHB accumulation in the media containing $VFA_{synthetic}$ or $VFA_{extracted}$, respectively.
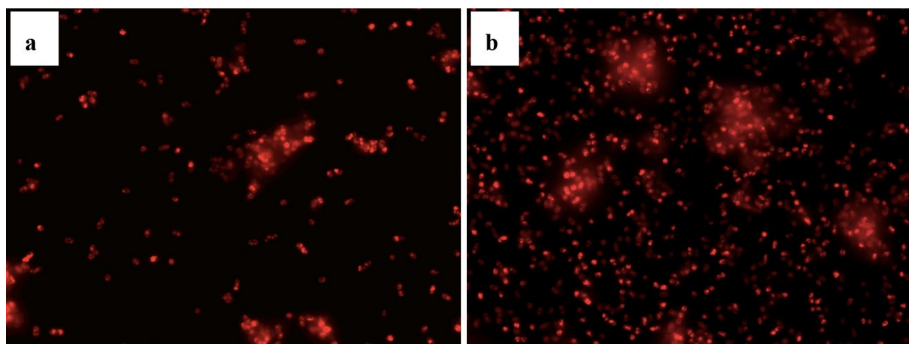

Fig. 3 PHA accumulation in *C. necator* DSM 13513 cells grown in LB medium supplemented with $VFA_{synthetic}$ (**a**; 24 h) or the $VFA_{extracted}$ mixture (**b**) observed by fluorescence microscopy (Axiovert 200 M, Zeiss, Göttingen, Germany) after 48 h of incubation at 30 °C, corresponding to the maximum production in the experimental conditions

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PHA production fed-batch fermentation experiments

Setup of fermentation conditions

On the basis of the batch culture results, to analyze the relationship between the carbon source, oxygen availability, and polymer accumulation, fed-batch fermentation experiments in LB medium supplemented with VFA$_{synthetic}$ were carried out under three different aeration conditions. Under no aeration, PHB production reached a value of 0.81% after 48 h (Table 1). Jackson et al. [28] reported that oxygen limitation increases the NADH/NAD ratio and that a high concentration of NADH inhibits citrate synthase and isocitrate dehydrogenase, blocking the TCA cycle. The accumulation of acetyl-CoA triggers PHB synthesis, during which PHB assumes the role of an alternative electron acceptor. Under steady aeration conditions, PHB production was lower (0.60% at 48 h, Table 1) than that without aeration. This result was probably due to PHB consumption related to the fermentative conditions when operating in fed-batch mode. When fermentation was performed by applying air sparging for 12 h, followed by no aeration, the highest PHB accumulation was recorded at 48 h (1.34%; Table 1), even though the microbial growth was lower (8.75 log CFU mL^{-1}) than that observed in previous fermentation tests with no aeration or with steady aeration (8.87 and 9.58 log CFU mL^{-1}, respectively). Variations in oxygen availability can lead to significant changes in the metabolism of *C. necator* cultures. These changes vary when different acid mixtures and substrates are used, affecting the synthesis of PHB in several ways [29]. The strategy for achieving efficient production of PHB by limiting the oxygen level together with intermittent feeding of a carbon source was investigated by Nath et al. [30] in a fed-batch culture of *Methylobacterium* sp. They reported a 0.8-fold increase in PHB production by limiting the oxygen levels in the fermenter. Instead, in this study, PHB production twofold higher than that in aeration conditions was achieved by limiting the aeration cycle to 12 h in the fed-batch mode. Although many studies have focused on the behavior of many microbial strains under different aeration conditions [30–32], the role of oxygen is not yet clear, since in some cases, oxygen allowed an enhancement in PHA synthesis, while in other cases, a negative influence was observed [30, 33, 34]. On the other hand, the results obtained demonstrate that the biotechnological performances of the bacterial strains in terms of the rate of production were strongly influenced by the composition of the growth medium as well as by the specific scale-up conditions of the biobased process.

PHA production in fed-batch fermentation with VFA$_{extracted}$

On the basis of the results obtained in the fermentation experiments with VFA$_{synthetic}$, air sparging for 12 h followed by no aeration was chosen to evaluate the accumulation of PHB with VFA$_{extracted}$. In this case, the culture accumulated a PHB percentage (0.52% at 48 h) lower than that in the previous experiment operating under the same aerating conditions (Table 1). The main difference among these experiments was the carbon source (VFA$_{synthetic}$ or VFA$_{extracted}$). In fact, the use of organic acids extracted from digestate could promote PHB accumulation instead of microbial growth, as also reported by Passanha et al. [35]. In contrast, the VFA$_{extracted}$ mixture used in this study stimulated microbial growth, achieving 9.59 log CFU mL^{-1}, a value higher than those obtained in previous fermentation tests. Moreover, the complex VFAs mixture extracted from digestate may include several nutrients and compounds that are taken up into the system in every feeding pulse, creating an imbalance

Table 1 Maximum PHB content (%) accumulated by *C. necator* DSM 13513 grown in LB medium supplemented with mixture of VFA$_{synthetic}$, VFA$_{extracted}$ in different cultivation conditions

Operating mode	Carbon source and aeration conditions		PHB content[a] (%)	Time (h)
[b]Batch culture	VFA$_{synthetic}$	Under shaken in aerobic condition	0.31 ± 0.001^E	24
	VFA$_{extracted}$	Under shaken in aerobic condition	0.48 ± 0.006^D	48
	Without VFA	Under shaken in aerobic condition	0.025 ± 0.002^F	24
[c]Fed-batch fermentation	VFA$_{synthetic}$	No aeration	0.81 ± 0.07^B	48
		Aeration	0.60 ± 0.01^C	48
		12 h of aeration	1.34 ± 0.02^A	48
	VFA$_{extracted}$	12 h of aeration	0.52 ± 0.03^{CD}	48

[a] The values represent the means $\pm$ SD of three replicates. Different letters indicate significant differences ($P < 0.05$)

[b] LB medium (600 mL) with 2% (v/v) of VFA$_{synthetic}$, VFA$_{extracted}$ or without VFA (control). Incubation at 30 °C for 24 or 48 h under shaken in aerobic condition at 200 rpm

[c] LB medium (4 L) with 2% (v/v) of VFA$_{synthetic}$ or VFA$_{extracted}$. pH at 6.5. Incubation at 30 °C for 48 h with agitation speed set at 200 rpm. Feeding at 2% (v/v) added at 3, 6, 9 and 12 h. Aeration: air sparging at 1 vvm; 12 h of aeration: air sparging at 1 vvm for 12 h followed by no aeration; no aeration

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between nutrient and carbon sources. In this way, the nutritional stress conditions necessary for PHB accumulation were lacking.

Monomer unit of the PHA polymer from VFA$_{synthetic}$ and VFA$_{extracted}$

Figure 4a shows the highest peak from the GC analysis of PHAs synthetized by *C. necator* DSM 13513 grown in the presence of the VFA$_{synthetic}$ mixture. The mass spectrum
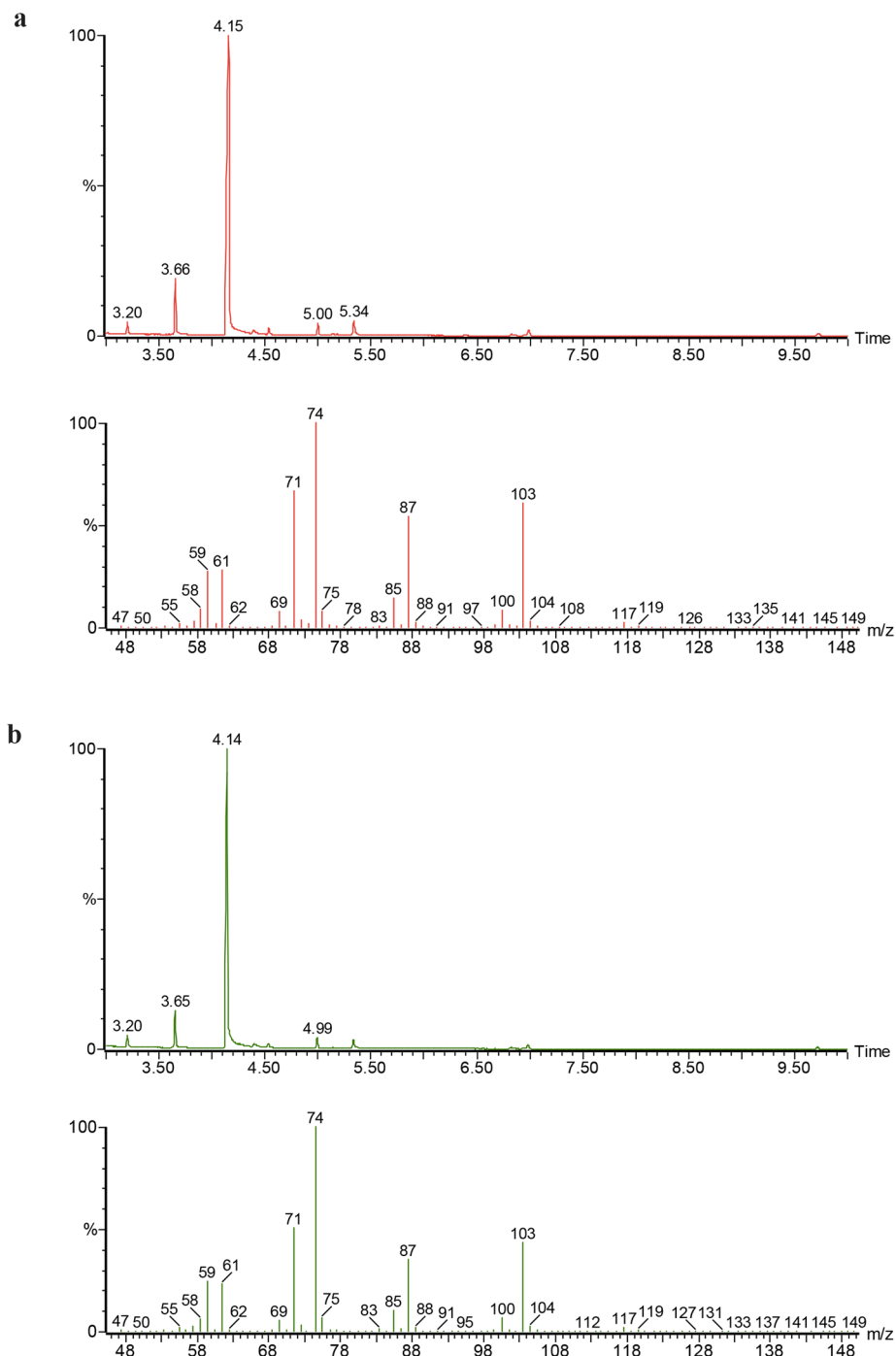

Fig. 4 GC/MS total-ion chromatograms and spectra of PHA monomers recovered by *C. necator* DSM 13513 grown with VFA$_{synthetic}$ (**a**) and the VFA$_{extracted}$ mixture (**b**)

of the chromatographic peak with a retention time of approximately 4.1 min, corresponding to the main monomer unit of the polymer, revealed the presence of 3-hydroxybutyrate, according to the mass spectral library from the NIST database. The other peaks derive from the solvents used for methanolysis. A similar result was obtained in the presence of VFA$_{extracted}$ (Fig. 4b). In fact, it has been shown that *C. necator* species predominantly accumulate the homopolymer PHB by the β-oxidation pathway [36] by using butyric acid, present in both VFA mixtures, which is well known as the main PHB precursor [37].

The mass spectra of both samples are dominated by the fragment at m/z=74 due to the McLafferty rearrangement of the methyl ester, whereas the fragment at m/z=103 is due to the break between carbon 3 and carbon 4 of the molecule. The loss of the CH$_3$OH group from the fragment at m/z=103 could instead explain the presence of the intense signal at m/z=71. The signal of the molecular ion shows low abundance, probably due to the high energy not absorbed by the molecule following electronic impact.

Structural characterization by NMR analysis

Figure 5 shows ^{1}H and ^{13}C NMR spectra of PHAs synthesized by *C. necator* DSM 13513 grown in the presence of the VFA$_{synthetic}$ mixture (A) and VFA$_{extracted}$ (B) compared with the poly(R)-3-hydroxybutyric acid standard. In the
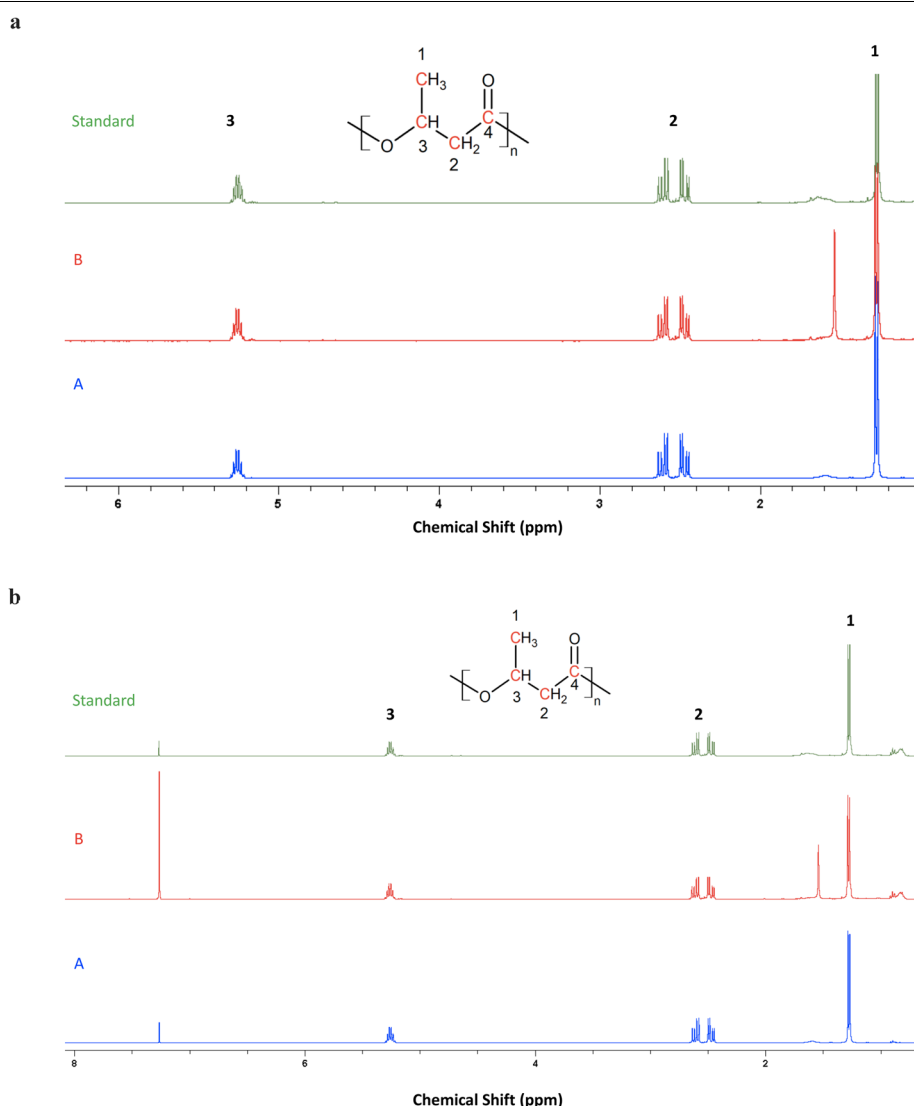

Fig. 5. ^{1}H NMR (**a**) and ^{13}C NMR (**b**) proton spectra of PHA from substrates with VFA$_{synthetic}$ (**a**), the VFA$_{extracted}$ mixture (**b**) and standard poly(R)-3-hydroxybutyric acid

¹H spectra, signals (Fig. 5a) were detected at chemical shifts of $\delta = 5.2$, 2.5, and 1.2, which corresponded to a −CH multiplet, −CH$_2$ multiplet, and −CH$_3$ doublet, respectively. The doublet at $\delta = 1.2$ was attributed to methyl protons (side chain of 3-hydroxybutyric acid). The multiplet at $\delta = 2.5$ ppm was due to the diastereotopic protons at position 2 of the chemical structure; finally, the quartet at $\delta = 5.2$ ppm was attributed to the proton close to the carboxyl oxygen (−CH). In the ¹³C NMR spectra (Fig. 5b), four signals were observed at chemical shifts of $\delta = 19.7$ ppm, 40.7 ppm, 67.6 ppm and 169.1 ppm, which were attributed to methyl carbon (side chain of 3-hydroxybutyrate), methylene carbon (backbone of 3-hydroxybutyrate), methane carbon (chiral center of 3-hydroxybutyrate) and carbonyl carbon, respectively. Finally, the NMR spectra indicate that the analyzed samples contained poly(R)-3-hydroxybutyric acid.

Characteristics of the bioplastic film
Thermal stability
Figure 6 shows the bioplastic disk obtained from the microbial cells of *C. necator* DSM 13513 grown on VFAs extracted from digested dairy wastewater effluent. The TGA mass loss curve and the corresponding derivative curve (DTGA) obtained for the studied PHB material

are shown in Fig. 7. The decomposition of PHAs showed a main weight loss from 200 °C to 250 °C followed by a moderate weight loss up to 499 °C [38]. The extrapolated onset temperature was 230.13 °C. The DTG curve shows three well-defined degradation stages at 247 °C and two
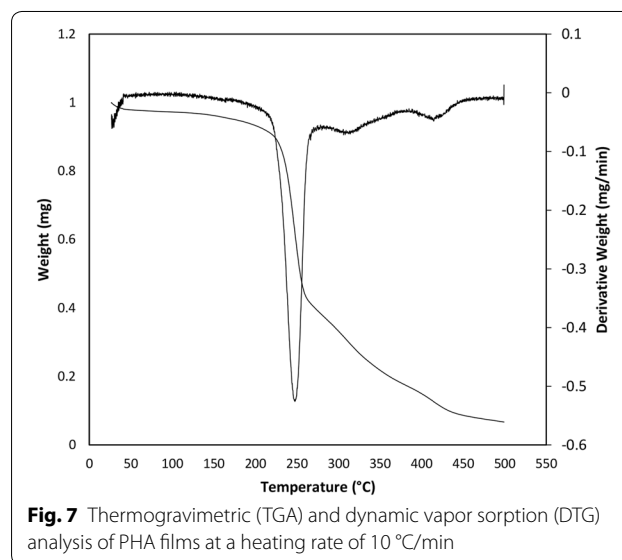
Fig. 7 Thermogravimetric (TGA) and dynamic vapor sorption (DTG) analysis of PHA films at a heating rate of 10 °C/min

Fig. 6 Bioplastic disk obtained from the microbial cells of *C. necator* DSM 13513 grown with the VFA$_{extracted}$ mixture from digested dairy wastewater effluent

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minor peaks at 376 °C and 414 °C, with the first one being the main transition as it comprises ca. 60 wt%. The first peak indicates the point of the greatest rate of change on the weight loss curve. As reported in the literature, PHA degradation occurs according to a random chain scission reaction of ester linkages just above their melting temperature, resulting in a TG curve characterized by a one-step process [38, 39]. Moreover, Herrera-Kao et al. [40] reported that although the degradation of PHB occurs in two stages, carboxylic acids and ester moieties were detected only in the former, suggesting that a random chain scission reaction takes place during thermal decomposition of this polymer. In the subsequent degradation, crotonic acid and a variety of oligomers may be further deconstructed into propylene, CO_2, acetaldehyde and ketene [41]. The additional thermal degradation that occurred at 376 and 414 °C has also been reported by Follain et al. [42] for PHBV commercial pellets. They reported that this additional degradation peak could be related to organic additives present in commercial samples. The degradation temperatures of the polymer were quite consistent with values reported in the literature for PHAs obtained from fermented sugar cane molasses (251 °C) [26] and slightly lower than those for PHBV at 4% HV (265 °C) and PHB at 0% HV (275 °C) [39].

Water vapor sorption isotherm of the PHA film

The isotherm curves, representing the water concentration at the sorption equilibrium state as a function of aw (%), of the PHA film are plotted in Fig. 8. The maximum mass gain of the film was below 20%, reflecting a medium affinity of the biopolymer to water. Hydrophilic ester groups could be responsible for water immobilization. A lower affinity to water was reported for PHA, PLA and PCL films [42–44].

The adsorption isotherm showed a sigmoidal curve that is typical of this type of biopolymer material. Nevertheless, the sigmoidal shape of the isotherms, as exhibited by the PHA films, is obviously maintained and corresponds to a type II isotherm in reference to Rogers' classification. Generally, in the literature, a sigmoid profile conforms to multimodal sorption divided into three stages. In the region of aw values between 0.3 and 0.7, water is absorbed at the multilayer, whereas at $a > 0.75$, the water absorbed corresponds to the condensation of water in the pores of the film. The casting method obviously favors the entrance of water molecules into the films. During solvent evaporation in the casting method, the polymer chains retain enough motion levels (such as translation and rotation motions) to create additional free volume, making the films less dense and more permeable.

The polymer structure is thus more easily opened and plasticized by water molecules, which behave as mobility enhancers during water sorption. This effect causes greater water sorption at high water activities (aw > 0.75). Sorption isotherm profiles can be graded according to the degree of crystallization. The BET molecular model of adsorption (fitted for a_w 0.3–0.5) [45], the GAB model and the Peleg model were used to describe the water adsorption of the films (Table 2). BET equation constants, which have a thermodynamic basis, have been used to analyze sample behavior and the interactions between components and water molecules. The
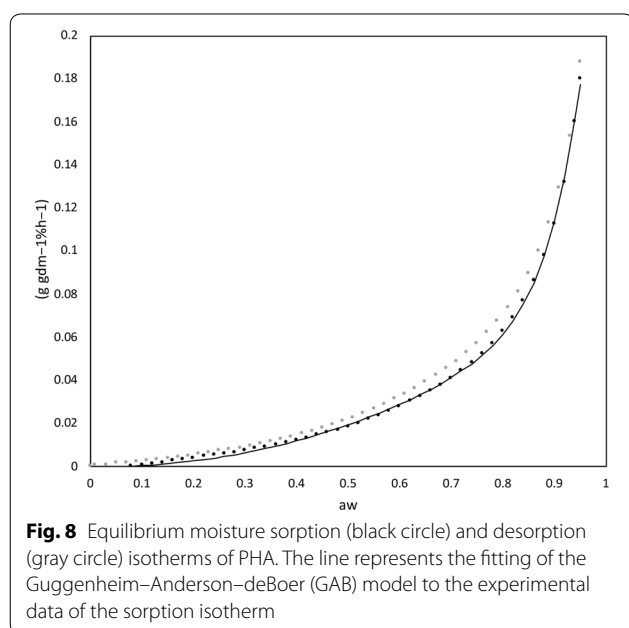
Fig. 8 Equilibrium moisture sorption (black circle) and desorption (gray circle) isotherms of PHA. The line represents the fitting of the Guggenheim–Anderson–deBoer (GAB) model to the experimental data of the sorption isotherm

Table 2 BET, GAB and Peleg parameters obtained from isotherms of PHA film at 40 °C

Model	Model parameters	Value
BET	m_0	0.022
	C	0.723
	RMSE	0.0001
	R^2 (%)	97.63
GAB	xm (g g_{dm}^{-1}%)	0.052
	C	0.003
	K	0.791
	RMSE	0.0036
	R^2 (%)	99.7
Peleg	k_1 (g g_{dm}^{-1}% h^{-1})	0.087
	k_2	0.214
	n_1	2.176
	n_2	14.929
	RMSE	0.0010
	R^2 (%)	99.97

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monolayer moisture content (x_m) of the PHA was 0.022 (mg mgd$_m$$^{-1}$), and the C constant and RMSE were 0.723 and 0.0001, respectively (Table 2). According to the GAB model, the x_m value is 0.052 (mg mgd$_m$$^{-1}$). This parameter is directly related to the number of adsorption sites. The greater degree of intermolecular interaction probably decreased the number of free sites for water molecules in the PHA polymeric matrix [46]. The parameters *C* and *K* are related to the quantity of water at the multilayer and to the energy needed to end the interaction. For the PHA film, the values are 0.003 and 0.79, respectively (Table 2). The root mean squared error (RMSE) is 0.0036. For the Peleg model, k_1 is 0.087 and k_2 is 0.214 (mg mgd$_m$$^{-1}$). The value of the constant k_1 is related to the mass transfer rate; e.g., the lower k_1 is, the higher the initial water absorption rate [47]. The maximum water absorption capacity is indicated by the k_2 constant; the lower k_2 is, the higher the water absorption capacity. The RMSE of the Peleg model for the PHA film is 0.0010. The Peleg and BET equations gave similar good fits that were better than that of the GAB model, on the basis of a comparison of RMSE values. Although the BET equation gave marginally better results than the Peleg and GAB equations, it was felt that the results justified the use of a limited a_w range (0.3–0.5).

Conclusions

Cupriavidus necator DSM 13513 was able to synthetize PHAs using a complex organic acid mixture extracted from a dairy wastewater biodigestion effluent as a carbon source. Structural characterization revealed the accumulation of 3-hydroxybutyrate by *C. necator* DSM 13513 cells. Bioplastic disks prepared by PHB showed physical properties similar to those of conventional plastics as good thermic properties and poor affinity to water. These results make the PHB obtained from digested dairy wastewater effluent suitable for specific biobased industrial applications.

Materials and methods
Bacterial strains and culture conditions
The microorganisms used in this study were *C. necator* DSM 13513, *C. necator* DSM 428 and *C. necator* DSM 531 (Leibniz Institute DSMZ, Braunschweig, Germany).

C. necator strains were routinely grown on LB medium containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl (pH 7.0). For solid medium, bacteriological agar (Oxoid S.p.A., Milan, Italy) was added at a concentration of 15 g L^{-1} and was dissolved by heating the medium. Strains were grown at 30 °C for 48–72 h under aerobic condition.

For PHA production, LB medium was supplemented with 2% (v/v) of a mixture of synthetic VFAs (VFA$_{synthetic}$)

or extracted VFAs (VFA$_{extracted}$). VFA$_{synthetic}$ is a synthetic mixture of pure organic acids composed by 0.6 g L^{-1} of acetic acid, 1.11 g L^{-1} of butyric acid and 0.5 g L^{-1} of propionic acid, used to simulate, in terms of VFAs, a real digestate effluent obtained in a previous study [11]. VFA$_{extracted}$ derived from digestate obtained from the anaerobic processing of a mixture of dairy wastewater from a mozzarella cheese factory. In this experiment, 6 L biodigesters (working volume of 5 L) were filled with a mixture of cheese whey and buttermilk (2:1 v/v) and inoculated with 5% (w/v) industrial animal manure pellets (Stalfert N$_2$; Organazoto Fertilizzanti S.p.A., Pistoia, Italy) [11]. After 30 days, the digestate was collected and centrifuged at 4010*g* for 10 min. The liquid fraction, containing the VFAs, was filtered (Minisart RC-25, 0.2 μm; Sartorius Stedim Biotech GmbH, Göttingen, Germany) and characterized by high-performance liquid chromatography (HPLC, refractive index detector 133; Gilson system; pump 307; column Metacarb 67 h from Varian with a 0.4 mL min^{-1} flow of 0.01 N H$_2$SO$_4$) [48] to determine its organic acid composition (acetic acid 4.17 g L^{-1} ± 0.31, propionic acid 4.11 g L^{-1} ± 0.54, lactic acid 3.97 ± 0.27 g L^{-1} and ethanol 20.24 g L^{-1} ± 0.52).

Screening by bacterial growth with VFAs
The bacterial strains were preinoculated by dissolving a single colony into 9 mL of LB medium and incubated overnight at 30 °C under aerobic condition. One milliliter of each culture (O.D.$_{600\ nm}$ 4.30) was inoculated in test tubes containing 10 mL of LB medium with 2% (v/v) VFA$_{synthetic}$ or VFA$_{extracted}$. From each tube, 220 μL of culture was dispensed in triplicate into a microtiter plate and incubated for 72 h at 30 °C in aerobic condition under shaking every 60 s. Growth curves were obtained by monitoring the O.D.$_{600\ nm}$ in a microplate reader (BioTek ELx808) every 30 min. LB medium without VFA was used as a control. All tests were performed in triplicate.

PHA production in batch culture with synthetic and extracted VFA
The preliminary screening based on the evaluation of bacterial growth ability in LB medium supplemented with VFAs led to the selection of the strain *C. necator* DSM 13513.

The PHA production of the selected bacterial strain was assessed in batch culture with LB liquid medium (600 mL) containing 2% (v/v) VFA$_{synthetic}$ or VFA$_{extracted}$. The flasks were inoculated with bacterial cultures as described above and incubated at 30 °C for 48 h under continuous shaking at 200 rpm. Flasks without VFA mixtures were used as controls. At the initial time and after 3, 6, 9, 24, 30 and 48 h, samples were withdrawn to detect

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the accumulation of PHAs in the cells by staining 1 mL of cell suspension with 1 drop of Nile Blue A [49]. Briefly, after incubation at 55 °C for 10 min and centrifugation at 1920*g* for 5 min, the pellet was washed with 0.9% NaCl solution and centrifuged again (1920*g* for 5 min). The excess stain solution was removed using 8% acetic acid for 1 min, and the pellet recovered from a new round of centrifugation was suspended in 0.9% NaCl [49]. The presence of PHAs in the cells was detected by fluorescence microscopy (Axiovert 200 M; Carl Zeiss Microscopy GmbH, München, Germany). Microbial growth was also evaluated by measuring $O.D._{600\,nm}$ (BioSpectrometer basic; Eppendorf s.r.l., Milan, Italy) and enumerating tenfold diluted cultures by the spread plate method on LB solid medium ($CFU\,mL^{-1}$).

For each sampling time, bacterial cultures grown in LB medium with $VFA_{synthetic}$ or $VFA_{extracted}$ were freeze-dried (Lyoquest -55; Telstar, Terrassa, Spain) for PHA extraction and quantification.

PHA extraction and quantification
For PHA extraction from *C. necator* DSM 13513 cells, a modification of the method proposed by Strazzullo et al. [50] was used. In detail, 100 mL of distilled water was added to a dry pellet (1 to 2 g) to obtain complete dispersion of the cells by ultrasonication (HK3300 ultrasonic sonicator cleaning bath; Falc Instruments s.r.l., Treviglio, Italy) for 20 min. SDS solution (10%; Serva Electrophoresis GmbH, Heidelberg, Germany) was added to digest the dispersed cells (wet cell weight:SDS ratio 1:1). After incubation for 1 h at 50 °C in a heater (MD-102 mixing block; Hangzhou Bioer Technology Co. Ltd, Hangzhou, China), the mixture was autoclaved for 20 min at 121 °C, cooled, transferred to tubes and centrifuged at 9500*g* for 30 min at 4 °C. The pellet was recovered and dried at room temperature.

PHB quantification was performed by using a K-HDBA kit (Megazyme Ltd., Bray, Ireland) to detect the D-3-hydroxybutyric acid concentration in the samples. The data were reported as PHB %, calculated as g PHB 100 g^{-1} of sample.

PHA production in fed-batch fermentation experiments
The fed-batch fermentation experiments were performed in a New Brunswick BioFlo 115 benchtop fermenter (Eppendorf s.r.l., Milan, Italy) with a working volume of 4 L and a pair of 6-blade Rushton impellers. The fermenter was filled with LB medium supplemented with 2% (v/v) $VFA_{synthetic}$ or $VFA_{extracted}$ and inoculated with 2% (v/v) *C. necator* DSM 13513 overnight culture as described above. The pH was automatically maintained at 6.5 by adding a solution of 4 M NaOH and setting the sensitivity for pH control at 0.10. A solution of 3% (v/v) Antifoam

204 (Sigma-Aldrich, Milan, Italy) was added after 1 h of fermentation to break any foam that had already formed. The experiments were performed at 30 °C for 48 h with an agitation speed of 200 rpm using three different aeration conditions: (1) air sparging at 1 vvm; (2) no aeration; (3) air sparging at 1 vvm for 12 h followed by no aeration. $VFA_{synthetic}$ or $VFA_{extracted}$ at 2% (v/v) was added at 3, 6, 9 and 12 h of the process to improve the maximum accumulation capability of the culture. Samples were withdrawn at 0, 3, 6, 9, 12, 24, 30 and 48 h to measure the optical density ($O.D._{600\,nm}$) and to enumerate bacterial cells ($CFU\,mL^{-1}$). For each sample, a subsample was freeze-dried (Lyoquest-55, Telstar, Terrassa, Spain) and used for PHB quantification as reported above.

Determination of monomer composition by gas chromatography/mass spectrometry (GC/MS)
The PHA monomeric composition was qualitatively analyzed by gas chromatography/mass spectrometry (GC/MS), and extracted freeze-dried PHAs were first subjected to methanolysis according to the method of Sathiyanarayanan et al. [51]. In detail, approximately 8 mg of PHAs was dissolved in 0.8 mL of chloroform, and then 0.8 mL of a methanol/sulfuric acid (85:15 v/v) mixture was added to the vials. After incubation at 105 °C for 210 min with shaking every 20 min, the solution was cooled at room temperature. Then, 0.4 mL of Milli-Q water was added, and the mixture was lightly vortexed for 1 min. The samples were centrifuged (Thermo Fisher Scientific, Waltham, USA) at 9500*g* for 2 min, and 200 μL of precipitated organic phase was mixed with crystalline sodium sulfate. One microliter of sample was injected into a GC/MS (Autosystem XL and Turbomass-Gold; PerkinElmer, Boston, USA) equipped with a Restek RTX-5MS WCOT 30 m × 0.25 mm column. The injector temperature was set at 250 °C; the initial isothermal temperature was 40 °C for 1 min, and then the temperature was increased to 120 °C at 15 °C min^{-1}, held for 2 min, and increased to 300 °C at 10 °C min^{-1}. Helium was used as the carrier gas at 1 mL min^{-1}. Mass spectra were obtained using electron impactionization (EI) with an energy of 70 eV at a frequency of 0.2 scan s^{-1} within the 50–600 m/z range. Identification of the mass signal was carried out by comparison with either standard compounds or molecular libraries such as NIST 05.

NMR characterization
Two milligrams of freeze-dried PHA was dissolved in 1 mL of deuterated chloroform ($CDCl_3$) containing 1% (v/v) tetramethylsilane. The mixture was stirred in a vortex, sonicated for 5 min, placed at 40 °C for 15 min to facilitate dissolution and transferred into a stoppered 5-mm NMR tube. NMR spectra were obtained

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with a 400-MHz Avance (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm Bruker Broadband Inverse (BBI) probe at ^{1}H and ^{13}C frequencies of 400.13 and 100.62 MHz, respectively, and at a temperature of 25 ± 1 °C (298 ± 1 K). The one-dimensional ^{1}H spectra were acquired with a 2-s thermal equilibrium delay, a pulse length of 90° between 7.82 and 7.9 µs (-2 dB of attenuation), 128 transients, 67,584 points in the time domain and 16 ppm (6410.3 Hz) as the spectral width. The one-dimensional ^{13}C spectra were acquired by the reverse ^{1}H–^{13}C decoupling technique. The spectra were acquired with a pulse length of 90° between 22 and 22.4 µs, 1200 transients, 32,768 points in the time domain and 250 ppm (25,125.629 Hz) as the spectral width. Spectra were processed by using Bruker TopSpin Software (v. 4.0.2). The free induction decays (FID$_s$) were Fourier transformed by applying an apodization of 0.3 Hz for ^{1}H and 1 Hz for ^{13}C experiments, and phase and basic corrections were applied to all spectra.

Formation and characterization of the plastic film
Biopolymer recovery
Cells of *C. necator* DSM 13513 were recovered by centrifugation (6080g for 10 min) after 48 h of incubation at 30 °C in LB medium supplemented with VFA$_{extracted}$. Microbial cells were freeze-dried and then suspended in chloroform (40 mL CHCl$_3$ g^{-1} dried cells). After incubation at 37 °C for 3–5 days, the solution was filtered (Minisart RC-25, 0.2 µm; Sartorius Stedim Biotech GmbH, Göttingen, Germany) to remove all undissolved material, and the filtrate was used to fill glass microplates. Finally, the chloroform was evaporated, allowing polymer recovery in the form of a thin bioplastic film [26].

Thermogravimetric analysis (TGA)
TGA was performed using a thermogravimetric analyzer (TGA 7/DZ; Perkin Elmer, Tokyo, Japan). Samples (3.0 ± 0.01 mg) were placed in aluminum pans inside the thermogravimetric balance and then heated under a dry nitrogen atmosphere (gas flow $= 20$ mL/min) in the range of 25–500 °C at a heating rate of 10 °C min. Two characteristic temperatures were collected, corresponding to the onset degradation temperature (To) and to the temperature of the largest peak in the first derivative curve of weight versus temperature (Tdp).

Dynamic vapor sorption analysis (DVS)
The absorption isotherms were measured using DVS (Q500 SA dynamic vapor sorption analyzer; TA Instruments, New Castle, USA). The main component was a microbalance with an accuracy of 0.1 g. The sample (5 to 9 mg) was placed in an aluminum capsule, and an empty aluminum capsule was used as a reference.

Measurements were conducted at 40 °C using the following procedure:

1. Conditioning: the samples were conditioned at 0% RH for the time necessary to reach a constant weight.

2. Absorption: the relative humidity was increased to 30%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90% and 95%. The sample remained in each step until a constant weight was reached.

From the curves relating to the percentage weight increase as a function of time, the absolute humidity (m) was calculated for the equilibrium of each water activity, expressed as milligrams of water absorbed at equilibrium on milligrams of dry substance.

The Brunauer, Emmett and Teller (BET), (Eq. (1)), Guggenheim–Anderson–deBoer (GAB) (Eq. (2), and Micha Peleg (Peleg) (Eq. (3) equations were used to describe the experimental data. These models are explained and rearranged as given below:

$$X = \frac{x_m \text{aw}}{(1 - \text{aw})(1 + C - 1) * \text{aw}} \tag{1}$$

$$X = \frac{x_m C K_{\text{aw}}}{(1 - K a_w)(1 - K a_w + C K a_w)}, \tag{2}$$

$$X = K_1 a w^{n1} + K_2 a w^{n2} \tag{3}$$

where X is the equilibrium moisture content (mg mgd$_m$$^{-1}$); x_m is the monolayer moisture content (mg mgd$_m$$^{-1}$); aw is the water activity; C and K are the GAB constants; and k_1, k_2, n_1 and n_2 are the Peleg model constants.

The goodness of fit of the mathematical models (Eqs. (1), (2), and (3)) was evaluated by means of the root mean squared error (RMSE):

$$RMSE = \sqrt{\frac{\Sigma(M_e - Mp)^2}{n}}, \tag{4}$$

where Me is the experimental value, Mp is the predicted value, and n is the number of data points.

Statistical analysis
One-way ANOVA followed by Tukey's HSD post hoc test for pairwise comparison of means (at $P < 0.05$) was used to assess the difference in the PHB percentage. Statistical analyses were performed using the SPSS 21.0 statistical software package (SPSS Inc., Cary, USA).

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