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

Giorgia Pagliano
Universita degli Studi di Napoli Federico II
Wanda Gugliucci
Universita degli Studi di Napoli Federico II
Elena Torrieri
Universita degli Studi di Napoli Federico II
Alessandro Piccolo
Universita degli Studi di Napoli Federico II
Silvana Cangemi
Universita degli Studi di Napoli Federico II
Fabio A Di Giuseppe
Universita degli Studi di Napoli Federico II
Alessandro Robertiello
Universita degli Studi di Napoli Federico II
Vincenza Faraco
Universita degli Studi di Napoli Federico II
Olimpia Pepe (olipepe@unina.it)
University of Naples Federico II
Valeria Ventorino
Universita degli Studi di Napoli Federico II

Research

Keywords: biodigestate, volatile fatty acids, Cupriavidus necator, poly-β-hydroxybutyrate, biopolymer properties

Posted Date: May 27th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-30604/v1

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

Giorgia Pagliano[a,1], Wanda Gugliucci[a,1], Elena Torrieri[a], Alessandro Piccolo[a], Silvana Cangemi, Fabio Angelo Di Giuseppe[a], Alessandro Robertiello[a], Vincenza Faraco[b], Olimpia Pepe[a,*], Valeria Ventorino[a]

[a] Department of Agricultural Sciences, University of Naples Federico II, Portici (Naples), Italy

[b] Department of Chemical Sciences, University of Naples Federico II, Naples, Italy

* Corresponding author at: University of Naples Federico II, Department of Agricultural Sciences, via Università 100, 80055 N, Portici (Naples), Italy. E-mail address: olipepe@unina.it (O.Pepe).

[1] These authors contributed equally to this work.

Abstract

Background: to establish bioplastics the real alternative to conventional plastics, high production costs have to be constrained by using different kind of wastewater streams as organic substrate and novel microbial strains as accumulating bacteria with high performance. Volatile fatty acids (VFAs) from effluent of dairy wastewater biodigestion represent a new and cheap feedstock used in this study for biopolymers production through microbial processes.

Results: *Cupriavidus necator* DSM 13513 was particularly able to accumulate PHAs operating in fed-batch mode by limiting oxygen level together with intermittent feeding of carbon source with the maximum PHB accumulated in 48 h without compromising the microbial growth. The complex VFA mixture from digestate did not influence the PHA homopolymer accumulation. In fact, structural characterization by NMR analysis revealed the 3-hydroxybutirrate synthesis (PHB) by *C. necator* DSM 13513 grown with these different VFAs mixtures. Moreover, the bioplastic disk obtained *C. necator*

DSM 13513 cells grown on VFA from digested dairy waste effluent, presented good thermic properties and low affinity to water.

Conclusions: overall results making the digested dairy waste effluent suitable for PHB production for specific bio-based 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 replace, progressively, plastics deriving from the fossil fuels with bio-based, biodegradable and compostable plastics deriving from no-food renewable sources [1]. The European Commission is promoting "Circular Economy Action Plan" to replace conventional plastic generally utilized for single-use products, with a bioplastic by 2021 (EUR-Lex-52018PC0340). In order to establish bioplastics as a real alternative to conventional plastics, high production costs have to be constrained by using different kind of wastewater streams as organic substrate and novel microbial strains as accumulating bacteria with high performance. This aim could be achieved by eco-designing sustainable biopolymers production through microbial bio-based process [2,3]. Among biopolymers, microbial polyesters known as polyhydroxyalkanoates (PHAs) are biodegradable plastics synthesized by different bacteria from a range of substrates including sugars and fatty acids [4]. Volatile fatty acids (VFAs) such as acetic, propionic, butyric, etc., are potentially renewable carbon sources [5] that could be used for biogas production [6] generation of electricity [7] and synthesis of PHAs [8]. However, the larger quantity of VFAs is in the effluents of the acidogenic bio-H_2 that represents an interesting feedstock for PHA production process and an opportunity to enhance the treatment of those effluents [9]. In this context, in the last decades, many studies focused the attention on energy source

such as hydrogen produced from dairy wastes [10,6] showing the options to valorize this effluent in the chemicals industry or by other biological system for energy recovery (e.g. methane). In fact, an interesting integrated system can be designed to combine energy source (mainly hydrogen) and biopolymers production (PHAs) utilizing the acid rich wastewater stream [4]. For these reasons, also the effluent from acidogenic digestion of dairy wastes is an inexpensive acid – rich wastewater stream for PHA production [11]. In particular, whey and buttermilk are suitable by–products that can be submitted to hydrolysis-acidification step under anaerobic condition to produce hydrogen and VFAs. In fact, this wastewater stream manly includes VFAs, lactic acid, alcohols and the residues which are un-hydrolyzed [9]. The resulted VFAs with lower carbons are the main precursors for the PHAs production by many microbial species.

A well-known PHA accumulating microbial species is *Cupriavidus* (*C*.) *necator*, synonym *Wautersia eutropha* and *Alcaligenes eutrophus*, formerly classified as *Ralstonia eutropha* [12]. Gram-negative bacterial strains belonging to this species are able to accumulate higher yield of PHAs from VFA 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 alkyl side chain present in the PHAs that is correlated to the substrate specificity of PHAs synthases. Typically, *C. necator* produce short chain (C3–C5) hydroxyalkanoic acids (PHA_{SCL}) which has an alkyl side chain such as P(3HB), P(3HV), P(4HB) and co–polymer of P(3HB–co–3HV) [14]. Therefore, it is essential to determine the chemical composition to evaluate their potential use for industrial purpose. Gas Chromatography-Mass Spectrometry (GC-MS) as well Nuclear Magnetic Resonance (NMR) are among the most widely used analysis for the chemical characterization of PHA structure [15] since their coupled use allow to obtain a careful and unambiguous molecular description.

PHAs represents a heterogeneous family of bio-based (co-)polyesters constituted by more than 150 different monomers to give materials with extremely different properties not always fully competitive

compared to conventional thermoplastics [16]. Although PHAs can show poor physical properties due to secondary crystallization and slow nucleation rate, they have physical properties comparable to petroleum-based thermoplastics. In particular, poli-β-idrossibutirrato (PHB) exhibits remarkable physical properties, comparable to polypropylene (PP) and polyethylene (PE). The stereo-chemical regularity of their 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 typical structures of pyrolysis products, i.e. crotonic acid and oligomers with a crotonate end group [18,19]. On the other hand, they have also shown that the parameters of melt processing must be optimized in order to avoid or restrict this phenomenon that reduces the processing window.

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

Results and Discussion

Ability of *C. necator* strains to growth in medium 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 presence of a mixture of VFAs. In the Figure 1 were reported the growth curves of the three strains in LB with $VFA_{synthetic}$ (Figure 1a), in LB with $VFA_{extracted}$ (Figure 1b) and LB (control; Figure 1c). All *C. necator* strains showed an enhanced growth by an excess of carbon source due to the organic acid addition ($VFA_{synthetic}$) in the culture medium. In particular, *C. necator* DSM 13513 showed the highest growth, achieving 1.97 $O.D._{600nm}$ at 40 h in the presence of $VFA_{synthetic}$ (Figure 1a) compared to the control assay (0.90 $O.D._{600nm}$; Figure 1c). These results demonstrated that these bacterial strains were well adapted to the acidogenic nutritional conditions due to the VFA mixture addition. The strain *C. necator* DSM 13513 showed the best growth (0.97 $O.D._{600nm}$; Figure 2b) also in presence of $VFA_{extracted}$ with a trend similar to that detected in LB broth (control tests without VFA; Figure 1c). Based on the chemical characterization of the $VFA_{extracted}$ mixture, the main difference with the $VFA_{synthetic}$ was the presence of the ethanol (20.24±0.52 g L^{-1} of ethanol) that did not affect the growth of the bacterial strains as showed by comparison with control test without VFA (Figure 1b and 1c). In fact, according to Obruca et al. [21] the exposition of *C. necator* to ethanol can enhance the PHB production without inhibit the microbial growth. Indeed, at low concentration organic acid mixture can be effectively utilized by *C. necator* as substrate for bacterial growth, although organic acid mixture could be toxic to cells [22].

This preliminary screening allowed to select the strain *C. necator* DSM 13513 for further investigations on PHA producing from VFA.

Batch culture experiments in media containing VFA

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 the PHAs synthesis. After 24 h of incubation at 30 °C at the up Log phase of growth, the PHB achieved 0.31% (Figure 2a) in the medium containing $VFA_{synthetic}$, 12 folds higher than that accumulated in the test control (optimal medium without $VFA_{synthetic}$) at same time (0.025%) (Figure 2c). In fact, various bacteria are able to utilize acetic, propionic and formic acid as substrate for PHA synthesis [23]. Among them, *C. necator* species is known to be one of the best PHB accumulating bacteria [24], even if the biotechnological performances are strain dependent. This was also demonstrated in this research since *C. necator* DSM 13513 was selected as the best strain to accumulate PHB from fatty acids effluent of anaerobic process fed with dairy wastes. After 24 h of growth also the cellular concentration of *C. necator* DSM 13513 was higher in the batch cultures with $VFA_{synthetic}$ (9.38 log CFU mL^{-1}; Figure 2a) with respect to the control medium (8.70 log CFU mL^{-1}; Figure 2c). The $VFA_{synthetic}$ 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 a consumption of PHB accumulated in the cells as energy reserve for lack of carbon source. Moreover, several microbial strains show PHB accumulation until the stationary phase as reported by Bhatia et al. [25] that studied recombinant *Escherichia coli* strain SKB99.

In presence of $VFA_{extracted}$, the strain *C. necator* DSM 13513 showed a particular trend prolonging the Log phase after 24 h of incubation ranging a microbial count from 7.37 log CFU mL^{-1} at 24 h to 9.03 log CFU mL^{-1} at 48h (Figure 2b). This behavior was related to an increase in the PHB production achieving a value 0.2% of PHB after 24 h of incubation with a significant increment of PHB until 0.48% at 48h (Figure 2b). Even if, *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 curve of PHB did not reach the plateau after 48 hours demonstrating the opportunity of extending accumulation time in the cells. However, at 48 h the same microbial concentration was observed in all experimental conditions.

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

PHAs accumulation in the media with and without VFAs mixtures was observed under fluorescence microscope as shown in the Figure 3. An increase of fluorescence was observed after 24 or 48 h of incubation, in correspondence with the maximum of PHB accumulation in the media containing $VFA_{synthetic}$ or $VFA_{extracted,}$ respectively.

PHA production fed-batch fermentation experiments

Set up of fermentation conditions

On the basis of the batch culture results, to analyze the relationship between carbon source, oxygen availability, and polymer accumulation, fed-batch fermentation experiments in LB supplemented with $VFA_{synthetic}$ were carried out testing three different aeration conditions. In no aeration condition, the 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 high concentration of NADH inhibits citrate synthase and isocitrate dehydrogenase, blocking the TCA cycle. The accumulation of acetyl-CoA triggers the PHB synthesis, during which the PHB assumes the role of an alternative electron acceptor. Under the steady aeration condition, the PHB production was lower (0.60% at 48 h, Table 1) than that produced without aeration. It was probably due to a PHB consumption related to the fermentative condition operating in fed-batch mode. When the fermentation was performed applying air sparging for 12 h followed by no aeration was recorded the highest PHB accumulation at 48 h (1.34%; Table 1), even if the microbial growth was lower (8.75 log CFU mL^{-1}) than that recovered in the previous fermentation 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 mixture and substrate are used affecting the synthesis of PHB in several ways [29]. The strategy for efficient production of PHB by limiting oxygen level together with intermittent feeding of carbon source was investigated by Nath et al. [30] in a fed batch culture of *Methylobacterium* sp. They reported an increase in PHB production by 0.8-fold by limiting the oxygen levels in the fermenter. Instead, in this study, a PHB production two-fold higher than aeration condition was achieved by limiting aeration cycle at 12 h in fed batch mode. Although there are many studies focused on the behavior of many microbial strains under different aeration condition [30,31,32], the role of oxygen is not already clear, since in some case allows an enhancement in PHA synthesis, in other cases a negative influence was observed [30,33,34]. On the other hands results obtained demonstrate that the biotechnological performances of the bacterial strains in terms of rate of production, was strongly influenced by composition of the growth medium as well as by the specific scale up conditions of the bio-based process.

***PHA production in fed-batch fermentation with* $\mathbf{VFA_{extracted}}$**

On the basis of 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 that previous experiment operating under the same aerating condition (Table 1). The main difference among these experiments is the carbon source ($VFA_{synthetic}$ and $VFA_{extracted}$). In fact, the use of organic acid extracted from digestate could promote the PHB accumulation instead of microbial growth as also reported by Passanha et al. [35]. On the contrary, the $VFA_{extracted}$ mixture used in this study stimulated the microbial grow achieving 9.59 Log CFU mL^{-1}, value higher than previous fermentation tests. Moreover, the complex nature of VFA mixture extracted from digestate may have several nutrients and compounds uptaken to the system in

every feeding pulse creating an in balance between nutrient and carbon sources. In this way, the nutritional stress conditions necessary for the PHB accumulation were lacking.

Monomer unit of the PHAs polymer from $VFA_{synthetic}$ and $VFA_{extracted}$

In the Figure 4a is shown the highest peak from the GC analysis of PHAs synthetized by *C. necator* DSM 13513 grown in presence of $VFA_{synthetic}$ mixture. The mass spectrum of the chromatographic peak with retention time about 4.1 min corresponding to the main monomer unit of polymer, revealed the presence of 3-hydroxybutyrate according to the mass spectral library from the NIST database. The other peaks depend from the solvents used for methanolysis. Similar result was obtained in the presence of $VFA_{extracted}$ (Figure 4b). In fact, it has been shown that *C. necator* species predominantly accumulates the homopolymer PHB by β-oxidation pathway [36] by using butyric acid, present in both VFA mixtures, that it 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 Mc Lafferty 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_3OH 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 the electronic impact.

Structural characterization by NMR analysis

The Figure 5 showed ^{1}H and ^{13}C NMR spectra of PHAs synthetized by *C. necator* DSM 13513 grown in presence of $VFA_{synthetic}$ mixture (A), and $VFA_{extracted}$ (B) compared with the Poly(R)-3-hydroxybutyric acid standard. In the ^{1}H spectra signals (Figure 5a) were detected at a chemical shift of δ= 5.2, 2.5, 1.2, which corresponded to a –CH multiplet, $-CH_2$ multiplet, $-CH_3$ doublet, respectively. The doublet at δ = 1.2 was attributed to the methyl protons (side chain of 3-hydroxybutyric acid). The multiplet at δ = 2.5

ppm was due to the diastereotopic protons at position 2 of the chemical structure, finally the quartet at δ = 5.2 ppm was attributed to the proton close to the carboxyl oxygen (-CH). In the ^{13}C NMR spectra (Figure 5b) were evidenced four signals at a chemical shift of δ= 19.7 ppm, 40.7 ppm, 67.6 ppm e 169.1 ppm, which were attributed, respectively, to methyl carbon (side chain of 3- hydroxybutyrate), methylene carbon (backbone of 3-hydroxybutyrate), methane carbon (chiral center of 3-hydroxybutyrate) and carbonyl carbon. At last, the NMR spectra indicate that the analyzed samples contained poly(R)-3-hydroxybutyric acid.

Characteristics of the bioplastic film

Thermal stability

The Figure 6 shows the bioplastic disk obtained from the microbial cells of *C. necator* DSM 13513 grew on VFA extracted from digested dairy wastes effluent. The TGA mass loss curve and the corresponding derivate curve (DTGA) obtained for the studied PHB is shown in Figure 7. The decomposition of PHAs showed mainly weight loss from 200°C to 250°C followed by a moderated weight loss up to 499 °C [38]. The extrapolated onset Temperature is 230.13 °C. The DTG curve shows three well-defined degradation stages at 247 °C, and two minor picks at 376 °C and 414 °C, being the first one the main transition as it comprises ca. 60 wt%. First peak indicates the point of greatest rate of change on the weight loss curve. As reported in literature, the PHAs degradation occurs according to a random chain scission reaction of ester linkage 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, only in the former, carboxylic acids and ester moiety were detected being possible suggest that a random chain scission reaction takes place during thermal decomposition of this polymer. In the following degradation the crotonic acid and a variety of oligomers may be further deconstructed into propylene, CO_2, acetaldehyde and ketene [41]. The additional thermal degradation occurred at values of

376 and 414 °C has also been reported by Follain et al. [42] for PHBV commercial pallet. They reported that this additional degradation peak could be related to organic additives present in the commercial samples. The degradation temperatures of the polymer were quite consistent with values reported in literature for PHAs obtained by fermented sugar cane molasse (251 °C) [26], and slight lower than PHBV at 4% HV (265 °C), and PHB at 0% HV (275 °C) [39].

Water vapor sorption isotherm of the PHAs film

The isotherm curves, representing the water concentration at the sorption equilibrium state as a function of aw (%) of the PHAs film are plotted in Figure 8. The maximum mass gain from film was below 20%, reflecting a medium affinity of biopolymer to water. The hydrophilic ester groups could be responsible of water immobilization. A lower affinity to water was reported for PHAs, PLA or PCL film [42,43,44]. The adsorption isotherm showed a sigmoidal curve that is typical for this type of biopolymer material. Nevertheless, the sigmoidal shape of isotherms, as exhibited by the PHAs 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 multi-mode sorption divided into three contributions. In the region of aw between 0.3 and 0.7 the 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 have retained 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 causes the greater water sorption at high water activities (aw > 0.75). A gradation of sorption isotherm profiles can be correlated 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 films (Table 2). BET equation constants, which have a thermodynamic base, has been used to analyze sample behavior and the interaction between the components and water molecules. The monolayer moisture content (x_m) of the PHAs was 0.022 (mg mgd_m^{-1}), the C constant and RMSE were respectively 0.723 and 0.0001 (Table 2). According to the GAB model, the x_m value is 0.052 (mg mgd_m^{-1}). This parameter was 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 PHAs polymeric matrix [46]. The parameter C and K are related to the quantity of water at the multilayer and to the energy needed to cut the interaction. For PHAs film are respectively 0.003 and 0.79 (Table 2). The root mean squared error (RMSE) are 0.0036. For the peleg model, k_1 was 0.087 and k_2 was 0.214 (mg mgd_m^{-1}). The constant value k_1 is related to mass transfer rate, e.g., the lower the k_1, the higher the initial water absorption rate [47]. The maximum water absorption capacity is reported by k_2 constant, the lower the k_2, the higher the water absorption capacity. The RMSE of Peleg model for PHA film are 0.0010. The Peleg and Bet equation gave similar good fits better than GAB model, comparing RMSE value. Although the Bet equation gave marginally better than the Peleg and GAB equation, it was felt that this was more than onset for using a limited a_w range (0.3-0.5).

Conclusions

Cupravidus necator DSM 13513 was able to synthetize PHAs using as carbon source the complex organic acids mixture extracted from a dairy wastewater biodigestion effluent. Structural characterization revealed the accumulation of 3-hydroxybutyrate by *C. necator* DSM 13513 cells used to prepare bioplastic disk that showed good thermic properties and poor affinity to water which are physical properties assimilating to conventional plastics. These results making the PHB obtained from digested dairy waste effluent suitable for specific bio-based industrial applications.

Material and Methods

Bacterial strains and culture condition

The microorganisms used in this study were *C. necator* DSM 13513, *C. necator* DSM 428 and *C. necator* DSM 531 (Leibniz Institute DSMZ-Germany).

C. necator strains were routinely grown on Luria-Bertani (LB) medium containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl (pH 7.0). For solid medium, bacteriological agar (Oxoid, 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.

For PHA production, LB medium was supplemented with 2% (v/v) of a mixture of synthetic VFA ($VFA_{synthetic}$) or extracted VFA ($VFA_{extracted}$). $VFA_{synthetic}$ is a synthetic mixture of pure organic acids (1:1:2 v/v of acetic, propionic and butyric acid, respectively) used to simulate, in terms of VFAs, a real digestate effluent obtained in previous study [11]. $VFA_{extracted}$ derived from the digestate obtained by the anaerobic process of a mixture of dairy waste from a mozzarella cheese factory. In this experiment, biodigesters (5 L) were filled with a mixture of cheese whey and buttermilk (ratio 2:1 v/v, respectively) and inoculated with 5% (w/v) of 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 x*g* for 10 min. The liquid fraction, containing the VFAs, was filtered (Minisart RC-25, 0.2 µL, Sartorius Stedim Biotech, Goettingen, Germany) and characterized by high-performance liquid chromatography (HPLC, refractive index detector 133; Gilson system; pump 307, column Metacarb 67 h from Varian with 0.4 mL min^{-1} flow of 0.01 N H_2SO_4) to determine its organic acids composition.

Screening by bacterial growth with VFAs

The bacterial strains were pre-inoculated dissolving a single colony into 9 mL of LB and incubated overnight at 30 °C. One mL of each culture ($O.D._{600nm}$ 4.30; 1% v/v) was inoculated in test tubes

312 containing 10 mL of LB medium with 2% (v/v) of $VFA_{synthetic}$ or $VFA_{extracted}$. From each tube, 220 µL of
313 culture were dispensed in triplicate into a microtiter plate and growth curves were obtained by monitoring
314 the $O.D._{600nm}$ in a Microplate Reader (BioTek ELx808) every 30 minutes for 72 h at 30 °C. LB without
315 VFA was used as control. All tests were performed in triplicate.
316
317 **PHA production in batch culture with synthetic and extracted VFA**
318 The preliminary screening for the evaluation of bacterial growth ability in LB supplemented with VFAs
319 allowed for the selection of the strain *C. necator* DSM 13513.
320 PHA production of the selected bacterial strain was assessed in batch culture with LB liquid medium
321 (600 mL) containing 2% (v/v) of $VFA_{synthetic}$ or $VFA_{extracted}$. The flasks were inoculated with 1% (v/v) of
322 bacterial cultures as above described and incubated at 30 °C for 48 h under continuously shaken at 200
323 rpm. Flasks without VFA mixture were used as control. At initial time and after 3, 6, 9, 24, 30 and 48 h,
324 samples were withdrawn to detect the accumulation of PHAs in the cells by staining 1 mL of cell
325 suspension with 1 drop of Nile Blue A [48]. Briefly, after incubation at 55 °C for 10 min and
326 centrifugation at 1920 x*g* for 5 min, the pellet was washed with 0.9% NaCl solution and centrifuged
327 again (1920 x*g* for 5 min). The excess of stain solution was removed using 8% acetic acid for 1 min and,
328 the pellet recovered by a new centrifugation was suspended in 0.9% NaCl [48]. The presence of PHAs
329 in the cells was detected by fluorescence microscope (Axiovert 200M, Zeiss, Göttingen, Germany). The
330 microbial growth was also considered by measuring $O.D._{600nm}$ (BioSpectrometer basic, Eppendorf, Milan,
331 Italy) and enumerating ten-fold diluted cultures by spread plate method on LB solid medium (CFU mL^{-1}).
333 For each sampling time, bacterial cultures growth in LB with $VFA_{synthetic}$ or $VFA_{extracted}$ were freeze-dried
334 (Lyoquest -55, Telstar, Terrassa, Spain) for the PHAs extraction and quantification.
335

PHA extraction and quantification

For the PHAs extraction from *C. necator* DSM 13513 cells, a modification of the method proposed by Strazzullo et al. [49], was used. In detail, 100 mL of distilled water were added to dry pellet (1 up to 2 g) in order to obtain a complete dispersion of the cells by ultrasonication (Ultrasonic sonicator cleaning bath HK3300, Falc, Treviglio, Italy) for 20 min. SDS solution (10%; Serva, Heidelberg, Germany) was added to digest the dispersed cells (wet cells weight:SDS ratio 1:1). After incubation for 1 h at 50 °C in a heater (Mixing block MD-102, Bioer, Hangzhou, China), the mixture was autoclaved for 20 min at 121°C, cooled, transferred to tubes and centrifuged at 9500 x*g* for 30 min at 4 °C. The pellet was recovered and dried at room temperature.

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

PHAs production fed-batch fermentation experiments

The fed-batch fermentation experiments were performed in a New Brunswick BioFlo 115 benchtop fermentor (Eppendorf, Milan, Italy) with a working volume of 4 L employing a pair of 6-blade Rushton impeller. Fermentor was filled with LB medium supplemented with 2% (v/v) of $VFA_{synthetic}$ or $VFA_{extracted}$ and inoculated with 2% (v/v) *C. necator* DSM 13513 overnight culture as above reported. The pH was automatically maintained at 6.5 by adding a solution of 4M NaOH setting the deadband for the pH control at 0.10. A solution of 3% (v/v) Antifoam 204 (Sigma-Aldrich, Milan, Italy) was added after 1 h of fermentation process to break a foam already formed. The experiments were performed at 30 °C for 48 h with an agitation speed set to 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 $VFA_{extracted}$ feeding 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 ($O.D._{600nm}$) and to enumerate bacterial cells (CFU mL^{-1}). For each sample, a sub-sample was freeze-dried (Lyoquest-55, Telstar) and used for PHB quantification as above reported.

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), freeze-dried PHAs extracted were first subjected to methanolysis according to the method of Sathiyanarayanan et al. [50]. In detail, about 8 mg of PHAs was dissolved in 0.8 mL of chloroform, and then 0.8 mL of methanol/sulfuric acid (85:15 v/v) mixture was added into the vials. After incubation at a 105°C for 210 min under shacking every 20 min, the solution was cooled at room temperature. Then 0.4 mL of milli-Q water was added and submitted slightly vortexed for 1 min. The samples were centrifuged (Thermofisher scientific, USA) at 9500 x*g* for 2 min, and 200 µL of precipitated organic phase was mixed with crystalline sodium sulphate. 1 µl of sample was injected into GC/MS PerkinElmer Autosystem XL (PerkinElmer, USA) Turbomass-Gold PerkinElmer, equipped with a Restek RTX-5MS WCOT, 30 m×0.25 mm column. The injector temperature was set at 250 °C, initial isothermal temperature was 40 °C for 1 min then increase to 120 °C at 15 °C min^{-1}, hold for 2 min, and increase to 300 °C at 10 °C min^{-1}. Helium was used as carrier gas at 1 mL min^{-1}. Mass spectra were obtained using electron impact ionization (EI) with energy of 70 eV at a frequency of 0.2 scan s^{-1} within the 50–600 m/z range. Identification of mass signal was carried out by comparing either with standard compounds or molecular libraries such as Nist 05.

NMR characterization

2 mg of freeze-dried PHA were dissolved in 1mL of deuterated chloroform (CDCl3) containing 1% (v/v) of tetramethylsilane. The mixture was stirred in a vortex, sonicated for 5 minutes, placed at 40 °C for 15

minutes to facilitate dissolution and transferred into a stoppered 5 mm NMR tube. NMR spectra were obtained with a magnet 400 MHz Avance (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm Bruker Broadband Inverse (BBI) probe, working at the ^{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 2 s of thermal equilibrium delay, a pulse length of 90° between 7.82 and 7.9 μs (−2 dB of attenuation), 128 transients, 67584 points in the domain of the time and 16 ppm (6410.3 Hz) as 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, 32768 points in the domain of the time and 250 ppm (25125.629 Hz) as spectral width. Spectra were processed by using Bruker TopSpin Software (v. 4.0.2). The free induction decays (FID_s) were Fourier transformed applying an apodization of 0.3 Hz for ^{1}H and 1 Hz for ^{13}C experiments respectively and phase and basic corrections were applied to all spectra.

Formation and characterization of plastic film

Biopolymer recovery

Cells of *C. necator* DSM 13513 were recovered by centrifugation (6080 x*g* 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) to remove all undissolved material, and the filtrate was used to fill glass microplates. Finally, chloroform was evaporated, allowing polymer recovery in the form of a thin bioplastic film [26].

Thermogravimetric Analysis (TGA)

TGA was performed using Thermogravimetric Analyzer, TGA 7/DZ, Perkin Elmer (Japan) equipment. Samples (3.0 ± 0.01 mg) were placed in aluminum pans inside the thermogravimetric balance and then heated under 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 extremum of the first derivate weight versus temperature peak (Tdp), respectively.

Dynamic vapor sorption analysis (DVS)

The absorption isotherms were measured using DVS (dynamic vaporization Q500SA, TA Instrument, 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, 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 a time necessary to reach a constant weight.

2. Absorption: the relative humidity has been increased and brought to 30%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90% and 95%. The sample remained in each step until the 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.

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

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

$$X = \frac{x_m CK_{aw}}{(1-Ka_w)(1-Ka_w+CKa_w)} \quad (2)$$

$$X = K_1 aw^{n1} + K_2 aw^{n2} \quad (3)$$

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

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

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

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

Statistical analysis

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

Abbreviations

PHAs: polyhydroxyalkanoates; VFAs: volatile fatty acids; PHB: polyhydroxybutyrate; SCL: short-chain-length; P(3HB): poly(3-hydroxybutyrate); P(3HV): poly(3-hydroxyvalerate); P(4HB): poly(4-

hydroxybutyrate); P(3HB-co-3HV): poly(3-hydroxybutyrate-co-3-hydroxyvalerate); GC-MS: Gas Chromatography-Mass Spectrometry; NMR: Nuclear Magnetic Resonance; PP: polypropylene; PE: polyethylene; TGA: thermogravimetric analysis; $VFA_{synthetic}$: synthetic volatile fatty acids; $VFA_{extracted}$: extracted volatile fatty acids; HPLC: high-performance liquid chromatography; DVS: dynamic vapor sorption analysis; RMSE: root mean squared error; GAB: Guggenheim-Anderson-deBoer; BET: Brunauer, Enmett and Teller; Peleg: Micha Peleg.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Author contributions

GP and WG performed microbiological experiments, analyzed data and drafted the manuscript. ET and FAG performed TGA and DVS analyses and drafted the manuscript for this part. AP and SC performed GC-MS and NMR analyses and drafted the manuscript for this part. AR performed fed-batch fermentation experiments. VF revised the manuscript. OP conceived the study, participated in its design and revised the manuscript. VV contributed to coordinate the study and revised the manuscript.

Acknowledgements

Not applicable

References

1. Guillard V, Gaucel S, Fornaciari C, Angellier-Coussy, H, Buche P, Gontard N. The next generation of sustainable food packaging to preserve our environment in a circular economy context. Front. Nutr. 2018;5:121.
2. Urtuvia V, Villegas P, González M, Seeger M. Bacterial production of the biodegradable plastics polyhydroxyalkanoates. Int. J. Biol. Macromol. 2014;70:208-213.
3. Pepe O. Microbial solutions to recover waste through a regenerative approach. EC Microbiology. 2018;14:778-779.
4. Pagliano G, Ventorino V, Panico A, Pepe O. Integrated systems for biopolymers and bioenergy production from organic waste and by-products: a review of microbial processes. Biotechnol. Biofuels. 2017;10:113.
5. Wang K, Yin J, Shen D, Li N. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: Effect of pH. Bioresour. Technol. 2014;161:395-401.

6. Pagliano G, Ventorino V, Panico A, Romano I, Robertiello A, Pirozzi F, Pepe O. The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk. J. Environ. Manage. 2018;217:110-122.
7. Chen Y, Luo J, Yan Y, Feng L. Enhanced production of short-chain fatty acid by co-fermentation of waste activated sludge and kitchen waste under alkaline conditions and its application to microbial fuel cells. Appl. Energy. 2013;102:1197-1204.
8. Oliveira C, Silva C, Carvalho G, Reis M. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities. New Biotechnol. 2017;37:69-79.
9. Kumar G, Ponnusamy V, Bhosale R, Shobana S, Yoon J, Bhatia S, Rajesh Banu J, Kim S. A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. Bioresour. Technol. 2019;287:121427.
10. Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens P, Esposito G. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Appl. Energy. 2015;144:73-95.
11. Pagliano G, Ventorino V, Panico A, Romano I, Pirozzi F, Pepe O. Anaerobic process for bioenergy recovery from dairy waste: meta-analysis and enumeration of microbial community related to intermediates production. Front. Microbiol. 2019;9:3229.
12. Vandamme P. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int. J. Syst. Evol. Microbiol. 2004;54:2285-2289.
13. Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr. Opin. Microbiol. 2010;13:321-326.
14. Liong MT. Beneficial microorganisms in agriculture, aquaculture and other areas. Switzerland: Springer International Publishers; 2015.

15. Muhr A, Rechberger E, Salerno A, Reiterer A, Malli K, Strohmeier K, Schober S, Mittelbach M, Koller M. Novel Description of mcl-PHA biosynthesis by *Pseudomonas chlororaphis* from animal-derived waste. J. Biotechnol. 2013;165:45-51.
16. Grassie N, Murray EJ, Holmes PA. The thermal degradation of poly(-(d)-β-hydroxybutyric acid): Part 1-Identification and quantitative analysis of products. Polym. Degrad. Stab. 1984;6:47–61.
17. Yeo J, Muiruri J, Thitsartarn W, Li Z, He C. Recent advances in the development of biodegradable PHB-based toughening materials: Approaches, advantages and applications. Mater. Sci. Eng. C. 2018;92:1092-1116.
18. Morikawa H, Marchessault RH. Pyrolysis of bacterial polyalkanoates. Can. J. Chem. 2006;59:2306–2313.
19. Kawalec M, Kurcok P, Kowalczuk M, Foltran I, Adamus G, Kurcok P, Scandola M. Carboxylate-induced degradation of poly(3-hydroxybutyrate). Biomacromolecules 2007;8:2–7.
20. Koller M, Maršalek L, Miranda de Sousa Dias M, Brainegg G. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New Biotechnol. 2017;37:24-38.
21. Obruca S, Marova I, Stankova M, Mravcova L, Svoboda Z. Effect of ethanol and hydrogen peroxide on poly(3-hydroxybutyrate) biosynthetic pathway in *Cupriavidus necator* H16. World J. Microbiol. Biotechnol. 2010;26:1261-1267.
22. Jaremko M, Yu J. The initial metabolic conversion of levulinic acid in *Cupriavidus necator*. J. Biotechnol. 2011;155:293-298.
23. Kalia VC, Raizaida N, Sonakya V. Bioplastics. J. Sci. Ind. Res. 2000;59:433-445.
24. Yun JH, Sawant SS, Kim BS. Production of polyhydroxyalkanoates by *Ralstonia eutropha* from volatile fatty acids. Korean J. Chem. Eng. 2013;30:2223-2227.

548 25. Bhatia S, Shim Y, Jeon J, Brigham C, Kim Y, Kim H, Seo H, Lee J, Kim J, Yi D, Lee Y, Yang Y.
549 Starch based polyhydroxybutyrate production in engineered *Escherichia coli.* Bioprocess Biosyst.
550 Eng. 2015;38:1479-1484.
551 26. Duque A, Oliveira C, Carmo I, Gouveia A, Pardelha F, Ramos A, Reis M. Response of a three-stage
552 process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer
553 composition. New Biotechnol. 2014;31:276-288.
554 27. Beccari M, Dionisi D, Giuliani A, Majone M, Ramadori R. Effect of different carbon sources on
555 aerobic storage by activated sludge. Water Sci. Technol. 2002;45:157–168.
556 28. Jackson F, Dawes E. Regulation of the tricarboxylic acid cycle and poly-hydroxybutyrate
557 metabolism in *Azotobacter beijerinckii* grown under nitrogen or oxygen limitation. J. Gen. Microbiol.
558 1976;97:303-312.
559 29. De Almeida A, Giordano A, Nikel P, Pettinari M. Effects of aeration on the synthesis of poly(3-
560 hydroxybutyrate) from glycerol and glucose in recombinant *Escherichia coli.* Appl. Environ.
561 Microbiol. 2010;76:2036-2040.
562 30. Nath A, Dixit M, Bandiya A, Chavda S, Desai AJ. Enhanced PHB production and scale up studies
563 using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. Bioresour. Technol.
564 2008;99:5749–5755.
565 31. Nikel PI, Pettinari MJ, Galvagno MA, Mèndez BS. Poly(3-hydroxybutyrate) synthesis by
566 recombinant *Escherichia coli arcA* mutants in microaerobiosis. Appl. Environ. Microbiol.
567 2006;72:614–2620.
568 32. Cavalheiro JMBT, De Almeida MCMD, Grandfils C, Da Fonseca MMR. Poly(3-hydroxybutyrate)
569 production by *Cupriavidus necator* using waste glycerol. Process Biochem. 2009;44:509–515.

570 33. Kim G, Lee I, Yoon S, Shin Y, Park Y. Enhanced yield and a high production of medium-chain-
571 length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by
572 combined use of glucose and octanoate. Enzyme Microb. Technol. 1997;20:500-505.
573 34. Borah B, Thakur PS, Nigam JN. The influence of nutritional and environmental conditions on the
574 accumulation of poly-ß-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. J. Appl. Microbiol.
575 2002;92:776–783.
576 35. Passanha P, Esteves S, Kedia G, Dinsdale R, Guwy A. Increasing polyhydroxyalkanoate (PHA)
577 yields from *Cupriavidus necator* by using filtered digestate liquors. Bioresour. Technol.
578 2013;147:345-352.
579 36. Da Cruz Pradella J, Ienczak J, Delgado C, Taciro M. Carbon source pulsed feeding to attain high
580 yield and high productivity in poly(3-hydroxybutyrate) (PHB) production from soybean oil using
581 *Cupriavidus necator*. Biotechnol. Lett. 2012;34:1003-1007.
582 37. Yu J, Si Y, Keung W, Wong W. Kinetics modeling of inhibition and utilization of mixed volatile
583 fatty acids in the formation of polyhydroxyalkanoates by *Ralstonia eutropha*. Process Biochem.
584 2002;37:731-738.
585 38. Li SD, He JD, Yu PH, Cheung MK. Thermal degradation of poly(3-hydroxybutyrate) and poly(3-
586 hydroxybutyrate-co-3-hydroxyvalerate) as studied by TG, TG-FTIR, and Py-GC/MS. J. Appl.
587 Polym. Sci. 2003;89:1530–1536.
588 39. Bordes P, Pollet E, Bourbigot S, Avérous L. Structure and properties of PHA/clay nano-
589 biocomposites prepared by melt intercalation. Macromol. Chem. Phys. 2008;209:1474–1484.
590 40. Herrera-Kao WA, Loría-Bastarrachea MI, Pérez-Padilla Y, Cauich-Rodríguez JV, Vázquez-Torres
591 H, Cervantes-Uc JM. Thermal degradation of poly(caprolactone), poly(lactic acid), and
592 poly(hydroxybutyrate) studied by TGA/FTIR and other analytical techniques. Polym. Bull.
593 2018;75:4191–4205.

41. Xiang H, Wen X, Miu X, Li Y, Zhou Z, Zhu M. Thermal depolymerization mechanisms of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Prog. Nat. Sci. 2016;26:58–64.
42. Follain N, Belbekhouche S, Bras J, Siqueira G, Marais S, Dufresne A. Water transport properties of bio-nanocomposites reinforced by *Luffa cylindrica* cellulose nanocrystals. J. Membr. Sci. 2013;427:218–229.
43. Follain N, Chappey C, Dargent E, Chivrac F, Crétois R, Marais S. Structure and barrier properties of biodegradable polyhydroxyalkanoate films. J. Phys. Chem. C. 2014;118:6165–6177.
44. Tenn N, Follain N, Soulestin J, Crétois R, Bourbigot S, Marais S. Effect of nanoclay hydration on barrier properties of PLA/montmorillonite based nanocomposites. J. Phys. Chem. C. 2013;117:12117–12135.
45. Peleg M. Assessment of a semi-empirical four parameters general model for sigmoid moisture sorption isotherms. J. Food Process Eng. 1993;16:21–37.
46. Yoshida CMP, Maciel VBV, Mendonça MED, Franco TT. Chitosan biobased and intelligent films: Monitoring pH variations. LWT Food Sci. Technol. 2014;55:83–89.
47. Turhan M, Sayar S, Gunasekaran S. Application of Peleg model to study water absorption in chickpea during soaking. J. Food Eng. 2002;53:153–159.
48. Ostle A, Holt J. Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. Appl. Environ. Microbiol. 1982;44:238-241.
49. Strazzullo G, Gambacorta A, Vella FM, Immirzi B, Romano I, Calandrelli V, Lama L. Chemical-physical characterization of polyhydroxyalkanoates recovered by means of a simplified method from cultures of *Halomonas campaniensis*. World J. Microbiol. Biotechnol. 2008;24:1513-1519.
50. Sathiyanarayanan G, Kant Bhatia S, Song HS, Jeon JM, Kim J, Lee YK, Kim YG, Yang YH. Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. Int. J. Biol. Macromol. 2017;97:710-720.

Figure Legends

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

Figure 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), $VFA_{extracted}$ mixture (b) and LB medium (c, control) during 48 h of incubation at 30 °C.

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

Figure 4. GC-MS total-ion chromatogram and spectrum of PHA monomers recovered by *C. necator* DSM 13513 grown with $VFA_{synthetic}$ (a), and $VFA_{extracted}$ mixture (b).

Figure 5. ^{1}H NMR (a) and ^{13}C NMR (b) proton spectrum of PHA from substrates with $VFA_{synthetic}$ (A), $VFA_{extracted}$ mixture (B) and standard Poly (R) -3-hydroxybutyric acid.

Figure 6. Bioplastic disk obtained from the microbial cells of *C. necator* DSM 13513 grown with $VFA_{extracted}$ mixture from digested dairy wastes effluent.

Figure 7. Thermogravimetric (TGA) and dynamic vapor sorption (DTG) analysis of PHA films at a heating rate of 10 °C / min.

Figure 8. Equilibrium moisture sorption (black circle) and desorption (grey circle) isotherm of PHA. Line represent the fitting of the Guggenheim-Anderson-deBoer (GAB) model to the experimental data of sorption isotherm.

Table 1. Maximum PHB (%) accumulated by *C. necator* DSM13513 grown in LB supplemented with mixture of $VFA_{synthetic}$, $VFA_{extracted}$ in different cultivation conditions.

Operating mode	Carbon source and aeration conditions		PHB (%)	Time (h)
***Batch culture**	$VFA_{synthetic}$	under shaken	0.31 ± 0.001^{E}	24
	$VFA_{extracted}$	under shaken	0.48 ± 0.006^{D}	48
	without VFA	under shaken	0.025 ± 0.002^{F}	24
§Fed-batch fermentation	$VFA_{synthetic}$	no aeration	0.81 ± 0.07^{B}	48
		aeration	0.60 ± 0.01^{C}	48
		12h of aeration	1.34 ± 0.02^{A}	48
	$VFA_{extracted}$	12h of aeration	0.52 ± 0.03^{CD}	48

*LB medium (600 mL) with 2% (v/v) of $VFA_{synthetic}$, $VFA_{extracted}$ or without VFA (control). Incubation at 30 °C for 48 h under shaken at 200 rpm.

§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; 12h of aeration: air sparging at 1 vvm for 12 h followed by no aeration; no aeration.

The values represent the means ± SD of three replicates. Different letters indicate significant differences ($P < 0.05$).

659
660
661
662

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

Figures

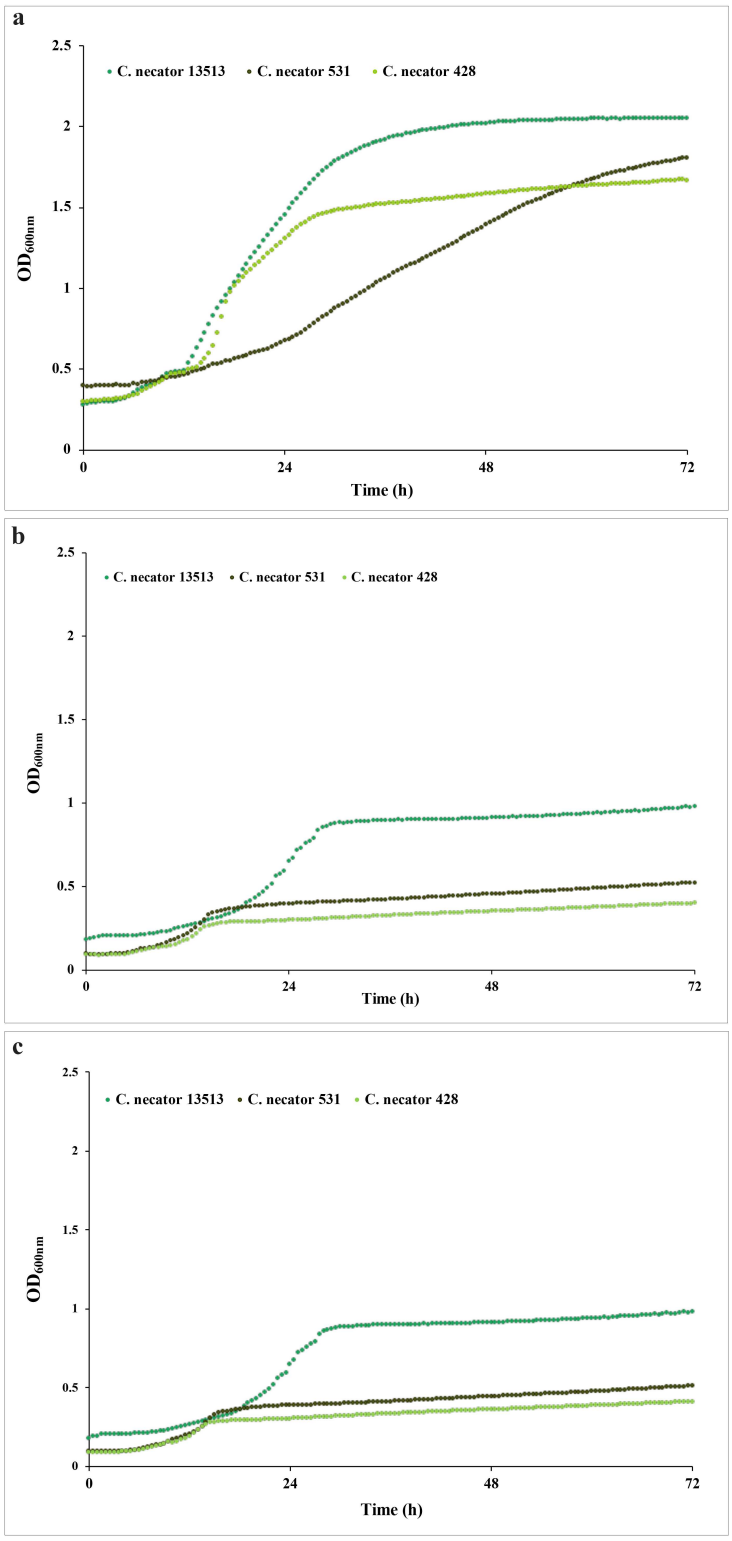

Figure 1

Microbial growth of Cupriavidus necator strains in LB medium supplemented with VFAsynthetic (a), VFAextracted mixture (b) and LB medium (c, control) during 72 h of incubation at 30 °C .

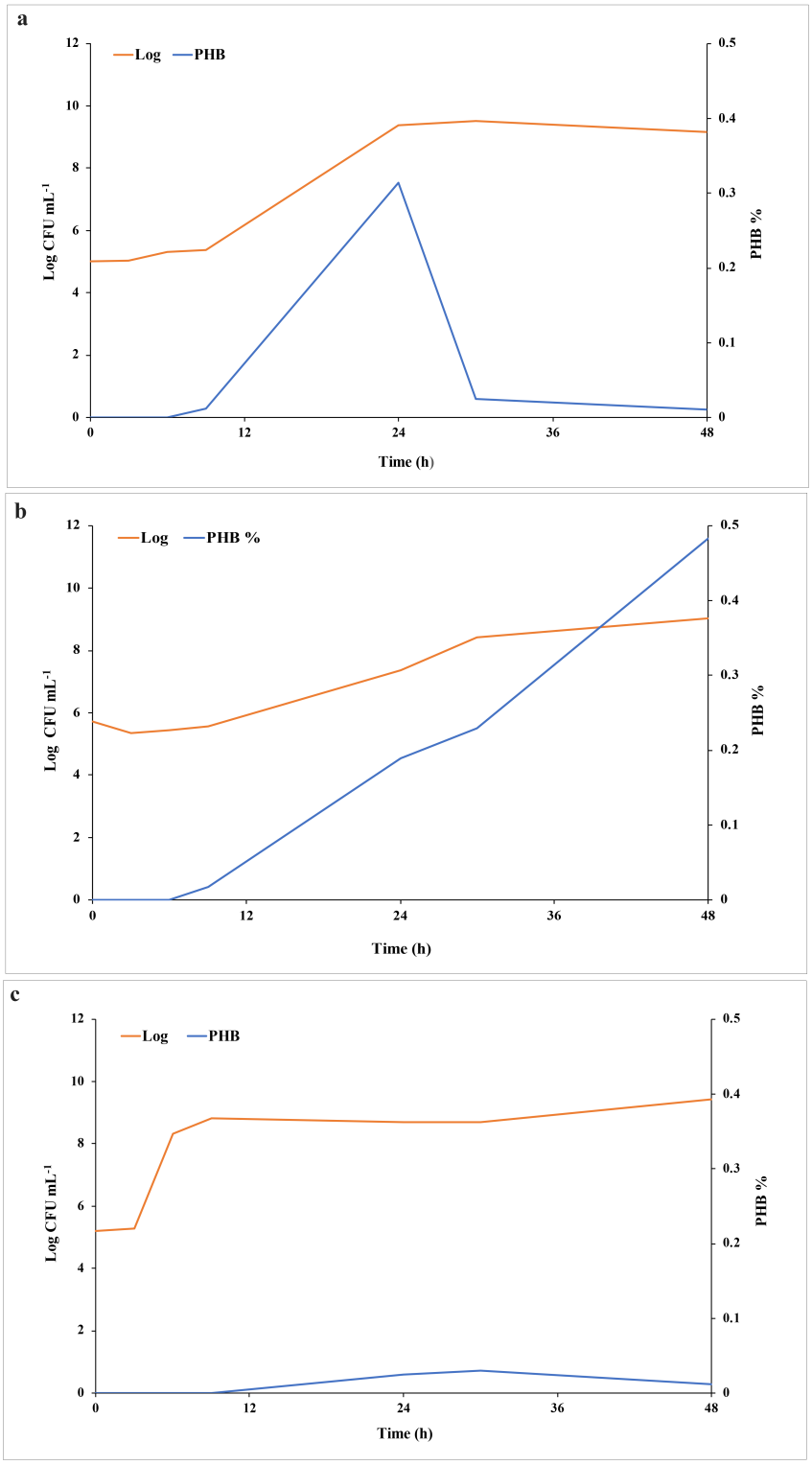

Figure 2

PHB production (%) and microbial counts (log CFU mL-1) of C. necator DSM 13513 in batch cultures grown in LB medium supplemented with VFAsynthetic (a), VFAextracted mixture (b) and LB medium (c, control) during 48 h of incubation at 30 °C.

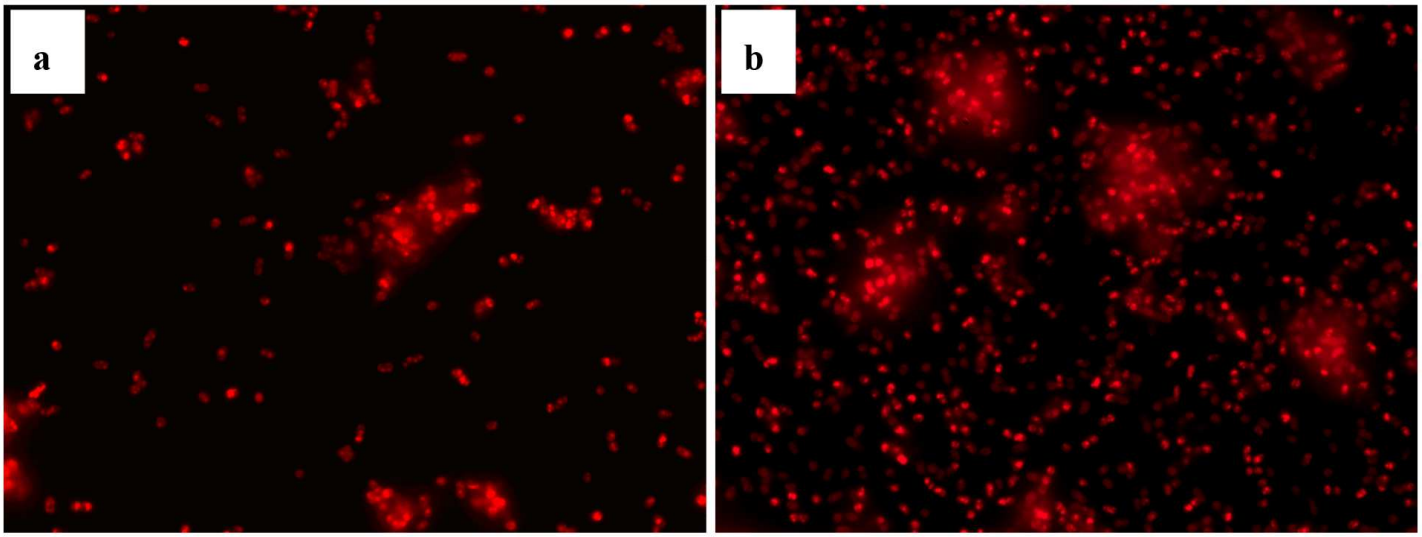

Figure 3

PHAs accumulation in C. necator DSM 13513 cells grown in LB medium supplemented with VFAsynthetic (a; 24h) or VFAextracted mixture (b) observed by fluorescence microscope (Axiovert 200M, Zeiss, Göttingen, Germany) after 48 h of incubation at 30 °C, corresponding to the maximum production in the experimental conditions.

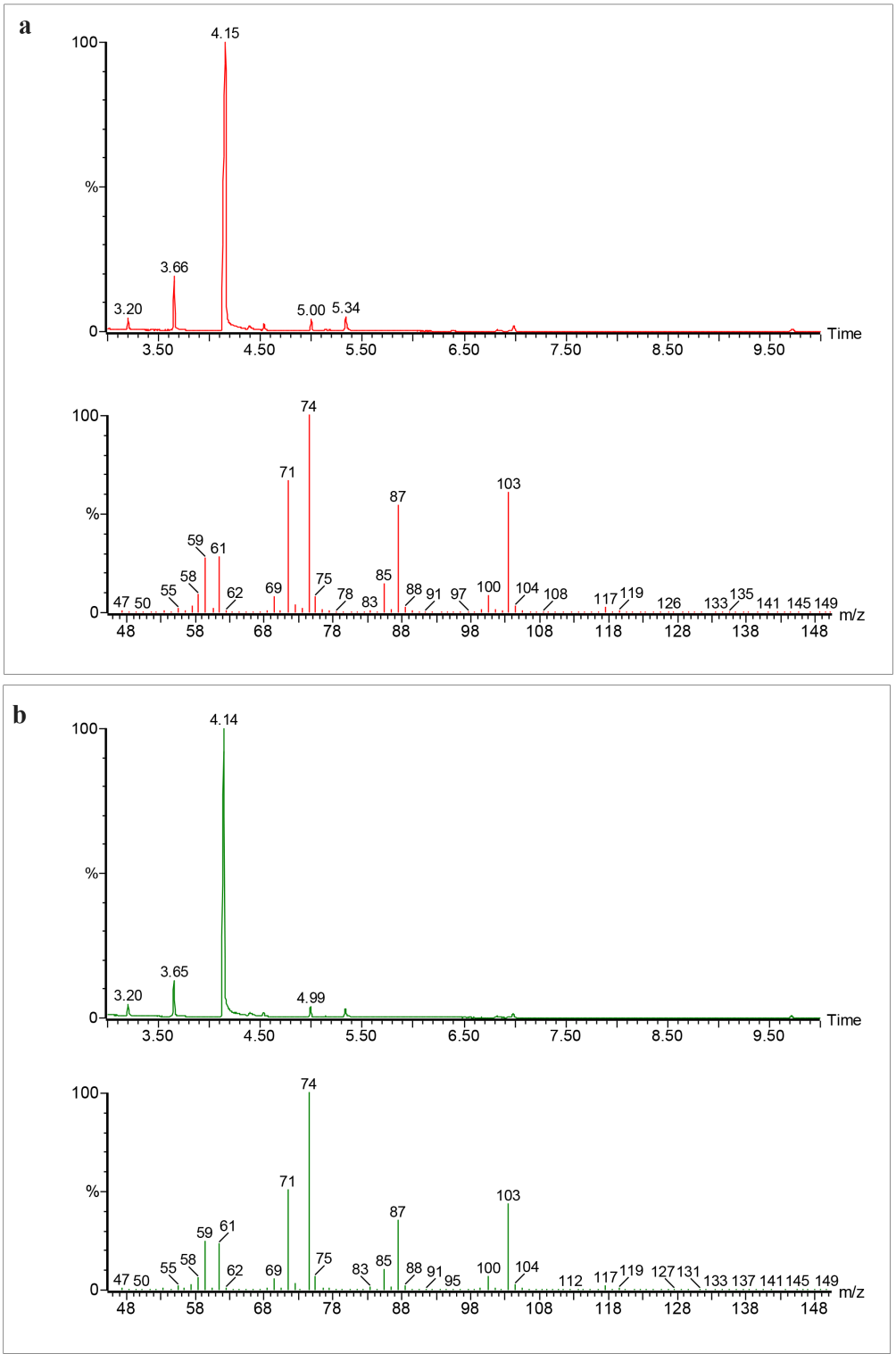

Figure 4

GC-MS total-ion chromatogram and spectrum of PHA monomers recovered by C. necator DSM 13513 grown with VFAsynthetic (a), and VFAextracted mixture (b).

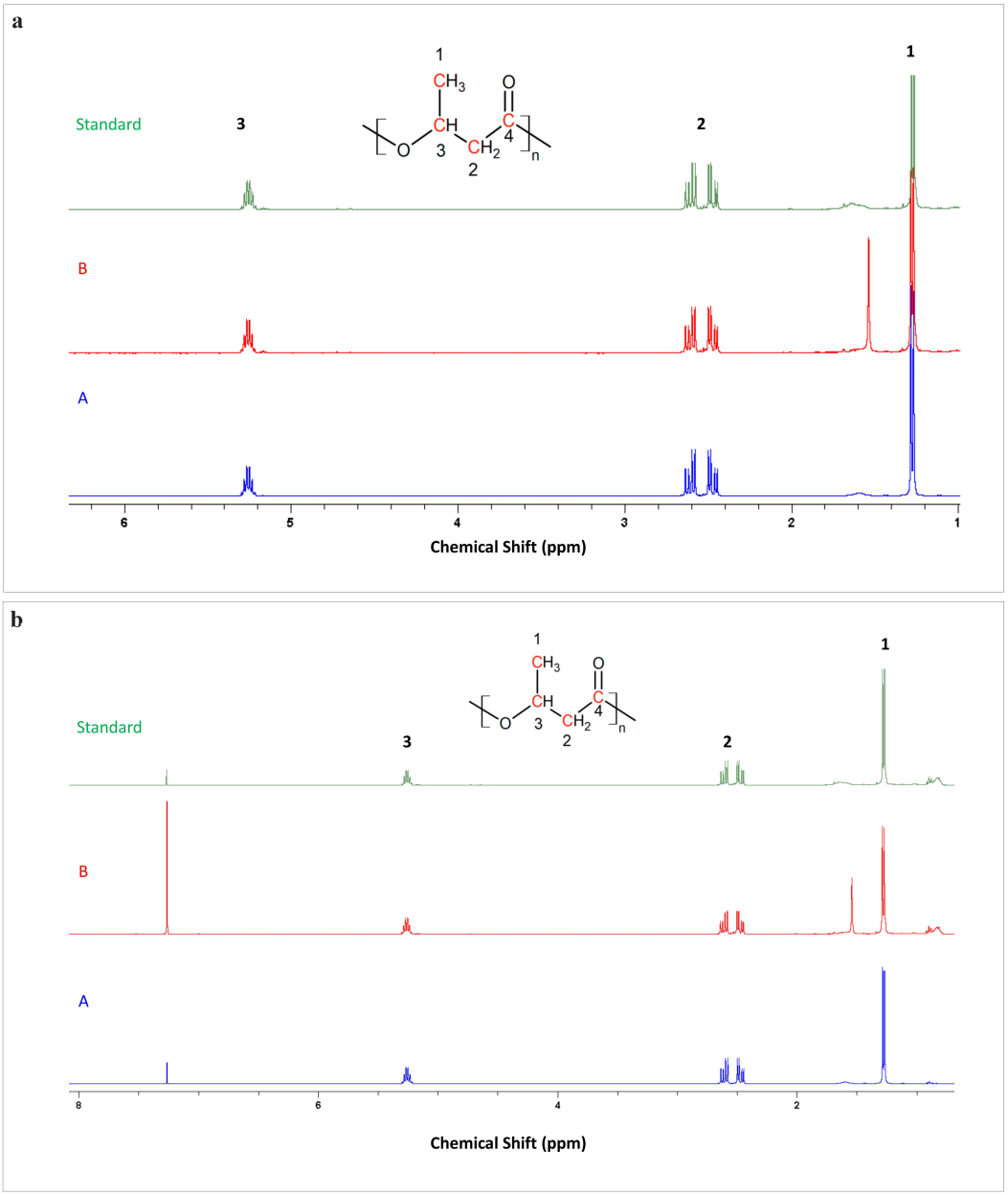

Figure 5

H NMR (a) and 13C NMR (b) proton spectrum of PHA from substrates with VFAsynthetic (A), VFAextracted mixture (B) and standard Poly (R) -3-hydroxybutyric acid.

Figure 6

Bioplastic disk obtained from the microbial cells of C. necator DSM 13513 grown with VFAextracted mixture from digested dairy wastes effluent.

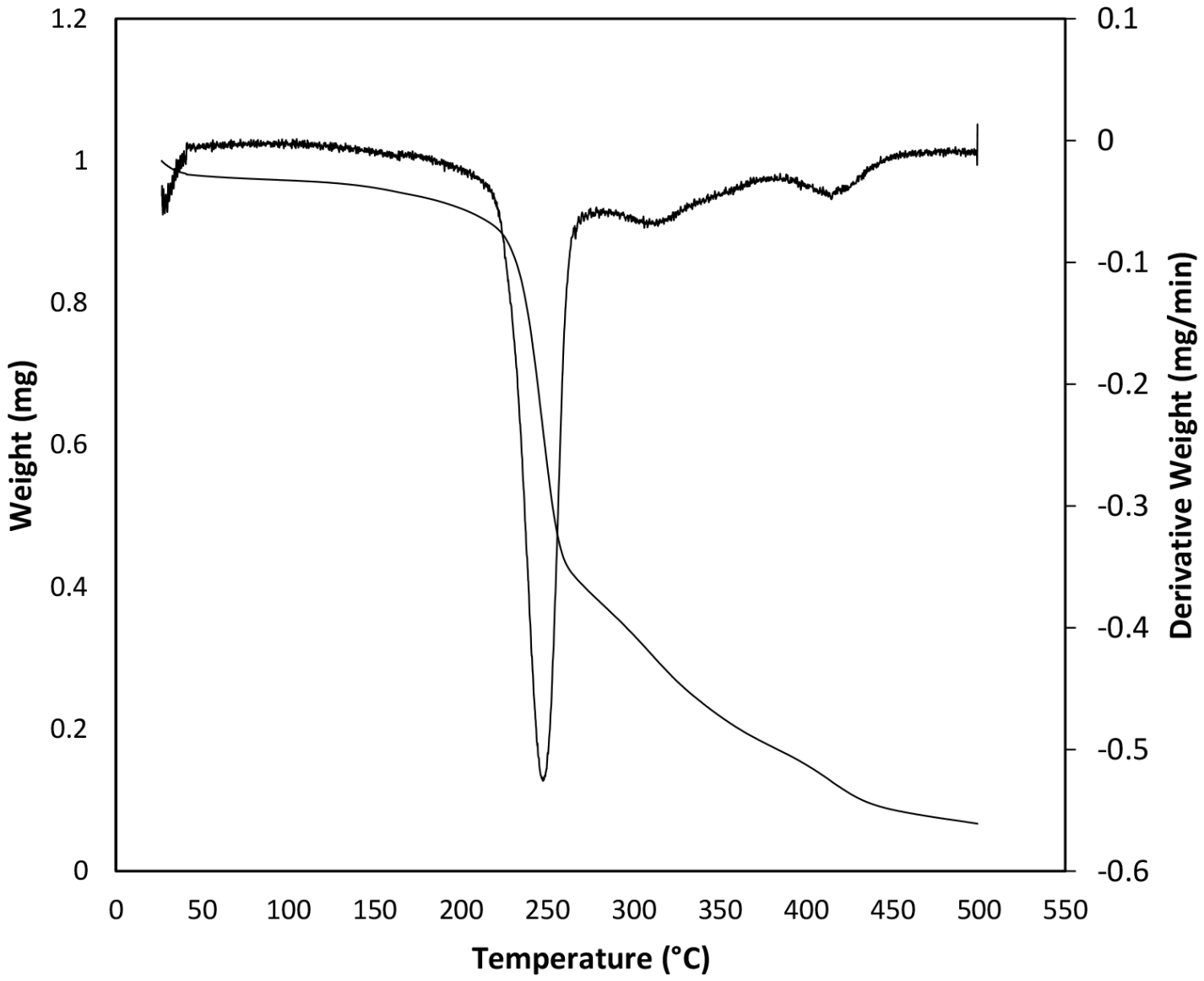

Figure 7

Thermogravimetric (TGA) and dynamic vapor sorption (DTG) analysis of PHA films at a heating rate of 10 °C / min.

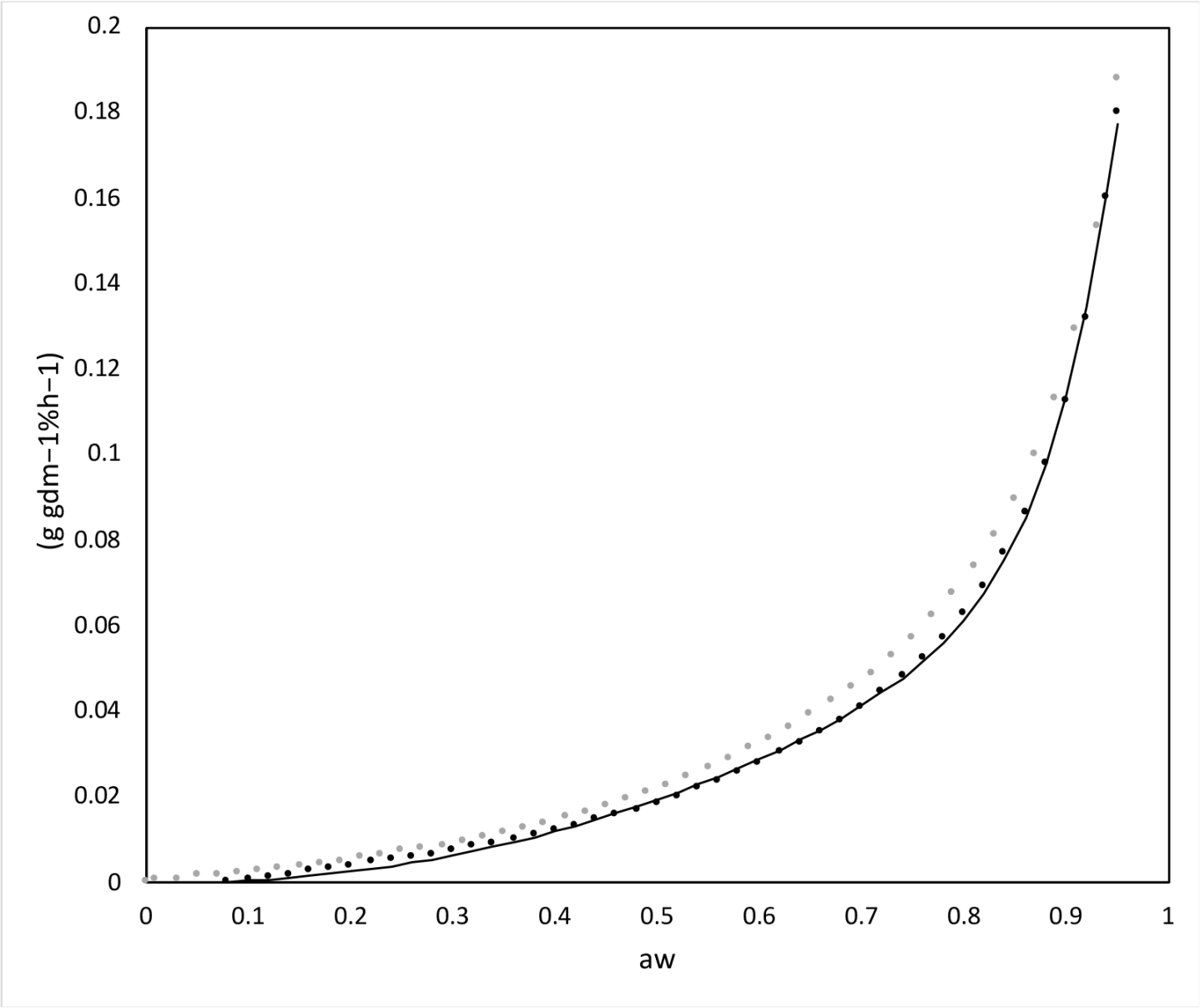

Figure 8

Equilibrium moisture sorption (black circle) and desorption (grey circle) isotherm of PHA. Line represent the fitting of the Guggenheim-Anderson-deBoer (GAB) model to the experimental data of sorption isotherm.