Università degli Studi di Padova

Padua Research Archive - Institutional Repository

Fast method for the determination of short-chain-length polyhydroxyalkanoates (scl-PHAs) in bacterial samples by In Vial-Thermolysis (IVT)

Original Citation:

Availability:

This version is available at: 11577/3252159 since: 2017-12-10T00:52:54Z

Publisher:

Elsevier B.V.

Published version:

DOI: 10.1016/j.nbt.2017.05.012

Terms of use:

Open Access

This article is made available under terms and conditions applicable to Open Access Guidelines, as described at <http://www.unipd.it/download/file/fid/55401> (Italian only)

(Article begins on next page)

Fast Method for the determination of Short-chain-length Polyhydroxyalkanoates (scl-PHAs) in Bacterial Samples by In Vial-Thermolysis (IVT)

F. Abbondanzi^{a*}, G. Biscaro^b, G. Carvalho^c, L. Favaro^d, P. Lemos^e, M. Paglione^f, C. Samori^{a, b}, and C. Torri^{a, b}

^a Interdepartmental Centre for Industrial Research Energy-Environment (CIRI EA), University of Bologna, via S. Alberto 163, 48123 Ravenna, Italy

^b Chemistry Department “Giacomo Ciamician”, University of Bologna, via Selmi 2, Bologna, Italy

^c UCIBIO, REQUIMTE, Chemistry Department, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal

^d Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE), University of Padua, Italy

^e LAQV. REQUIMTE, Chemistry Department, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal

^f National Research Council (CNR), Institute of Atmospheric Sciences and Climate (ISAC), Via Gobetti 101, Bologna, Italy

* Corresponding author. Tel.: +39 (0)544 937373; fax: +39 (0)544 937411.

e-mail address: federica.abbondanzi@unibo.it

Abstract

A new method based on the GC-MS analysis of thermolysis products obtained by treating bacterial samples at high temperature (above 270°C) has been developed. This method, here named “In-Vial-Thermolysis” (IVT), allowed for the simultaneous determination of short-chain-length polyhydroxyalkanoates (scl-PHA) content and

composition. The method was applied to both single strains and microbial mixed cultures (MMC) fed with different carbon sources. The IVT procedure provided similar analytical performances compared to previous Py-GC-MS and Py-GC-FID methods, suggesting a similar application for PHA quantitation in bacterial cells. The results from the IVT procedure and the traditional methanolysis method were compared; the correlation between the two datasets was fit for the purpose, giving a R^2 of 0.975. In searching for further simplification, the rationale of IVT was exploited for the development of a “field method” based on the titration of thermolyzed samples with sodium hydrogen carbonate to quantify PHA inside bacterial cells. The accuracy of the IVT method was fit for the purpose. These results open up the possibility for the on-line measurement of PHA productivity. Moreover, they allow for the fast and inexpensive quantification/characterization of PHA for biotechnological process control, as well as investigation over various bacterial community and/or feeding strategies.

Keywords

Polyhydroxyalkanoates, Thermolysis-GC-MS, Thermolysis-titration, Crotonic acid, Mixed Microbial Cultures (MMC)

Highlights

- In-Vial Thermolysis (IVT) allows for GC-MS determination of scl-PHA amount and composition.
- IVT is fast, simple, and does not require special lab equipment.
- Direct titration of the IVT products allows for scl-PHA quantification without a GC instrument.

1 Introduction

Polyhydroxyalkanoates (PHAs) are a family of intracellular polyesters. They are synthesized by a wide variety of prokaryotic microorganisms, as carbon and energy storage material, under stress conditions including nitrogen and/or phosphate restriction, as well as excess carbon [1, 2]. Biodegradable PHAs have a broad range of applications; in particular, the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (P(3HB-3HV)) has a high potential for the substitution of conventional plastics because it has thermoplastic properties comparable to those of petroleum-based polyolefins, such as polypropylene and polyethylene [3, 4].

Single strains and, more recently, mixed microbial cultures (MMC) have been increasingly investigated in order to reduce PHA production costs using feedstocks originating from various wastes, such as: fermented molasses [5], glycerol [6], cheese whey [3, 7, 8], olive oil mill wastewater [9], pyrolysis oil [10] and slaughterhouse by-products [11]. Efficient PHA-storing organisms have been selected in these processes applying feast and famine (FF) cycles [12, 13], where organic carbon is provided to the biomass during a short feast phase, followed by a long period of starvation (famine phase).

In order to reduce the research cost in bioreactors production related with adaptation of the process regime and the changes in feeding strategy, an accurate and fast monitoring of PHA amount and composition is crucial [14]. Many analytical techniques are under investigation and some improvements have been achieved: from optical and electronic microscopy to spectroscopy and biosensors [15, 16, 17, 18]. It has also been demonstrated that the pyrolysis products derived from PHA can be used as molecular

71 markers to identify and quantify PHA in bacterial biomass [19]. However, this approach
72 coupled with GC analysis (Py-GC) has not yet been fully-exploited in this field.

73 The present study aims at developing a new analytical approach that could simplify and
74 accelerate the real-time monitoring of bacterial cultures able to produce short chain
75 length-PHA (scl-PHA, hereafter named PHA). The goal was to improve the previous
76 pyrolysis-based method developed by Torri et al (2014) [19]. The method presented
77 here, following the QuEChERS (**Quick, Easy, Cheap, Effective, Rugged, Safe**)
78 philosophy [20, 21, 22, 23, 24], focused on the simplification of the quali/quantitative
79 analysis of PHA in bacterial biomass. Moreover, since bacterial lipid metabolism is
80 closely related to the synthesis of reserve macromolecules such as PHA [25], the
81 developed protocol could also be used for the qualitative estimation of long chain fatty
82 acids (LCFA) within bacterial membranes.

83 Specifically, the method presented here, termed “IVT” (In-Vial Thermolysis) was based
84 on the GC-MS analysis of the thermolysis products obtained from high temperature
85 treatment (above 270°C) of both single strain bacteria and MMC. This method allows
86 for the simultaneous determination of PHA content and PHA-monomer composition.

88 **2 Materials and Methods**

89 All solvents and chemicals used in this study were obtained from Sigma-Aldrich
90 (purities $\geq 98\%$) and used without purification. Standard poly(3-hydroxybutyrate)
91 P(3HB) was purchased from Biomer® (DE). The copolymer P(3HB-3HV) (**3HB/3HV**
92 **80 mol%/20 mol%**) was obtained as previously reported in the literature [26].

2.1 Microbial samples (Single strain and Microbial Mixed Cultures)

Cupriavidus necator DSM 545 was grown in shake-flasks according to Samorì et al 2015 [26] with four different feeding substrates: i) glucose; ii) glucose and valeric acid; iii) glucose, valeric acid and γ -butyrolactone; iv) acetic and propionic acid (Table 1). An assortment of MMC samples (MMC1-14, Table 1) were obtained from different sequencing batch reactors adapted to a feast/famine feeding regime, as described in various studies [5, 6, 12, 26], with different feeding substrates: glycerol, acetic acid, propionic acid, fermented cheese whey, bio-oil and triethyl citrate. The amount of PHAs in each bacterial sample was determined according to the standard methanolysis procedure reported in literature [27].

Table 1. Microbial samples used in the present work: single strain or MMC, feeding substrates and intracellular PHA content.

Entry	Sample name	Single strain /MMC	Feeding substrate	3HB mol% : 3HV mol%	PHA in CDM (Wt%) ^a
1	Sing 1	<i>C. necator</i>	Glucose	100:0	74.0 $\pm$ 2.0
2	Sing 2	<i>C. necator</i>	Glucose + Valeric acid	80:20	54.0 $\pm$ 2.0
3	Sing 3	<i>C. necator</i>	Glucose + Valeric acid + γ -butyrolactone	90:10	64.0 $\pm$ 1.2
4	Sing 4	<i>C. necator</i>	Acetic+ Propionic acid	81:19	64.0 $\pm$ 3.2
5	MMC 1	MMC	Glycerol	100:0	20.5 $\pm$ 2.2
6	MMC 2	MMC	Glycerol + Methanol	95:5	23.4 $\pm$ 2.5
7	MMC 3	MMC	Fermented biooil	94:6	17.5 $\pm$ 1.8
8	MMC 4	MMC	Fermented cheese whey	89:11	5.0 $\pm$ 0.4
9	MMC 5	MMC	Fermented cheese whey	85:15	5.0 $\pm$ 0.6

10	MMC 6	MMC	Fermented cheese whey	90:10	8.0 ± 0.9
11	MMC 7	MMC	Fermented cheese whey	88:12	13.0 ± 1.5
12	MMC 8	MMC	Acetic+ Propionic acid	91:9	44.0 ± 9.0
13	MMC 9	MMC	Acetic+ Propionic acid	89:11	43.0 ± 8.2
14	MMC 10	MMC	Acetic+ Propionic acid	90:10	3.0 ± 0.4
15	MMC 11	MMC	Acetic+ Propionic acid	89:11	37.0 ± 8.0
16	MMC 12	MMC	Acetic+ Propionic acid	90:10	40.0 ± 8.7
17	MMC 13	MMC	Acetic+ Propionic acid + Triethyl citrate	98:2	24.0 ± 6.0
18	MMC 14	MMC	Acetic+ Propionic acid + Triethyl citrate	99:1	8.0 ± 0.7

^a Determined by methanolysis

107
108

2.2 IVT of microbial samples

Freeze dried microbial samples (5 mg) or standard P(3HB) (2-3 mg) were transferred to in a screw cap vial (4 ml volume, 50 mm high). The vial was left with the cap off for 5 min in order to reduce the electrostatic behavior of lyophilized powder, allowing for the gentle deposition of all powder to the bottom of the vial. Subsequently, as shown in Figure 1, the vial was closed and then placed on a hot plate at 350°C. During this procedure, the bottom of the vial reached a temperature of 300-320°C, whereas the upper part of the vial and the cap did not exceed 80°C. For safety reasons and in order to avoid cap damage or the release of thermolysis vapors, the temperature was carefully monitored during method setup (e.g. by means of an infrared (IR) thermometer).

Different treatment times (20, 30, 60 and 120 min) were tested, in order to investigate the thermolytic process kinetics. Since thermolysis was completed within 20 min, this time was chosen for the final set up of the IVT procedure.

After 20 min, the vials were removed from the hot plate and left to cool down to room temperature before adding the internal standard (2-ethyl-butanoic acid, 0.25 mg dissolved in water). The sample was then diluted with CH₃CN (4 ml) and subjected to silylation-GC-MS analysis (see below). In the titration method, the sample was directly subjected to titration with 5 µM NaHCO₃ solution containing 1 mg/ml of bromothymol blue.

Finally, an aliquot of the CH₃CN-solution (0.1 ml) was withdrawn and subjected to silylation and GC-MS analysis for the determination of PHAs and fatty acid profiles and amount. For this purpose, *N,O* bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane (0.1 ml) and pyridine (0.02 ml) were added to the sample and the solution was heated at 60°C for 30 min. The identification of trimethylsilylated products

was confirmed by direct GC-MS analysis with the polar GC column and method previously used for Py-GC-MS [19]. The GC-MS analyses were performed with a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 mm film thickness), with helium as carrier gas (at constant pressure, 33 cm s⁻¹ linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s⁻¹ within the 12-600 m/z range. The following thermal program was used: 50°C hold for 10 min, and then increased up to 325°C at 10°C min⁻¹.

Figure 1.

2.3 Calibration

The amount of PHA in the biomass samples was determined by GC-MS from the quantity (Q_{AC}) of the most abundant 2-alkenoic acid (AC) derived from the thermolysis of the corresponding monomer in the polymer chain. The following 2-alkenoic acids were used for quantitation:

(*E*)-but-2-enoic acid (crotonic acid)

(*E*)-pent-2-enoic acid (pentenoic acid)

The quantity Q_{AC} in the thermolysate was calculated from the following formula:

$$Q_{AC} = Q_{IS} \cdot A_{AC} / A_{IS}$$

where Q_{IS} is the mass of the internal standard (2-ethylbutanoic acid) expressed in mg, while A_{AC} and A_{IS} are the GC-MS peak areas of the AC and internal standard, respectively.

The relationship between the quantity Q_{AC} of the selected 2-alkenoic acid and the corresponding quantity of the monomer in the sample ($X_{HA} \cdot Q_{PHA}$) was determined using the calibration curve obtained by treating standard polymers of known monomer composition, where X_{HA} is the mass fraction of the monomer in the polymer.

The ratio Q_{AC}/Q_{PHA} corresponds to the yield (Y_{AC}) of AC of the entire analytical procedure. The best linear relationship was determined by the least square fitting method:

$$Q_{PHA} = a(Q_{AC}) + b$$

For the fatty acid determination, a semi-quantitative analysis was performed by calculating the relative area of each fatty acid in comparison to the total area of all fatty acids.

3 Results and Discussion

3.1 IVT of purified PHA samples

The chromatogram obtained by applying low temperature thermolysis to the homopolymer P(3HB) and the copolymer P(3HB-3HV)) showed the typical alkenoic acid markers already found during pyrolysis (Figure 2) [19]. The mass spectra of the main peaks obtained from P(3HB) were characterized by the base peak at m/z 143 (loss of methyl radical from the molecular ion), an intense peak at m/z 69 ($C_4H_5O^+$) and a peak at m/z 99 ($C_5H_{11}Si^+$), in agreement with literature data [14]. The most abundant peak was attributed to the *trans* isomer according to previous studies pertaining to the analytical

pyrolysis of P(3HB) [17, 18]. Similar to pyrolysis, when P(3HB-3HV) was submitted to thermolysis, an additional peak was detected, likely identified as (*E*)-pent-2-enoic acid. A relevant yield of a homologue series with intense peaks at m/z 69 ($C_4H_5O^+$), 143 and 155 was also found, likely identified as P(3HB) oligomers. For P(3HB-3HV), four peaks were attributed to dimers whereas several peaks were attributed to various oligomers, in agreement with the possible monomeric sequence expected in the polymer. The same yield of 2-butenic acid and 2-pentenoic acid from low temperature IVT was obtained ($27 \pm 5\%$ w/w_{PHB} or w/w_{PHV}, determined by 20 replicates), significantly lower than that obtained through pyrolysis ($40\text{--}50\%$ w/w_{PHA}). This was probably related to an increased production of PHA oligomers due to specific reaction conditions (e.g. slow heating). Different amounts of P(3HB) were subjected to IVT in order to evaluate the linearity between the quantity of the 2-alkenoic acid produced (Q_{AC}) and the amount of the treated sample in the 0.2 – 5 mg range. Q_{AC} of 2-butenic acid was approximately linear with the quantity of P(3HB) subjected to IVT ($n=12$, $R^2 = 0.956$).

Figure 2.

3.2 IVT of microbial samples

The typical PHA markers obtained by treating P(3HB-3HV) – enriched microbial biomass with IVT were not qualitatively influenced by the non-PHA cellular material, as already observed with pyrolysis (Figure 3).

Moreover, it was reasonable to assume that the low temperature of thermolysis treatment minimized the depolymerization of proteins and carbohydrates, thus avoiding significant analytical interferences. The chromatograms of IVT-treated microbial

biomass were qualitatively similar to those of IVT-treated PHA, with a lower contribution of PHA oligomers.

In addition, a noticeable amount of long chain fatty acids (LCFA) could be detected in the 20-25 minute portion of chromatogram (Figure 3). The mass spectra of each trimethylsilyl (TMS) fatty acid peak was characterized by the base peak corresponding to each molecular ion (a part from C14:1, C14:0-OH and C17:1), an intense peak corresponding to the loss of a methyl group from the molecular ion and a peak at m/z 117, typical of all fatty acids (Table 2).

Figure 3.

215 Table 2. TMS derivatives of carboxylic acids (fatty acids and alkenoic acids) from the
 216 IVT of the microbial biomass. Bold characters refer to the most abundant peaks.

Source	Compound ^a	Retention	
		time (min)	Main peaks (m/z)
P(3HB), P(3HB-3HV)	2-butenic acid	8.51	143 , 99, 75
P(3HB-3HV)	2-pentenoic acid	10.28	117 , 113, 75
P(3HB), P(3HB-3HV)	(E)-3-(but-2-enoyloxy)butanoic acid	15.80	143 , 75, 69
P(3HB-3HV)	(E)-3-(pent-2-enoyloxy)butanoic acid	16.75	157, 143, 69
Lipids	C14:0	21.41	300, 285 , 228
Lipids	C14:1	21.60	283 , 143, 129
Lipids	C15:0	22.04	314, 299 , 143
Lipids	C16:1	22.80	326, 311 , 254
Lipids	C14:0-OH	22.86	373 , 331, 233
Lipids	C16:0	22.98	328, 313 , 256
Lipids	C17:0	23.64	342, 327 , 145
Lipids	C17:1	23.78	325 , 129, 117
Lipids	C18:2	24.52	352, 337 , 229
Lipids	C18:1	24.60	354, 339 , 129
Lipids	C18:0	24.77	356, 341 , 145

^a trimethyl silylated derivatives. The tentative identification was based on a combination of MS library and fragment mass.

The IVT method gave similar analytical performances compared to the Py-GC-MS and Py-GC-FID methods [19], suggesting the same application for PHA quantification in bacterial cells. In order to quantitatively evaluate the matrix effect, the results from the IVT procedure were compared to those from the traditional methanolysis approach (Figure 4). The correlation between the two datasets was slightly lower than that observed with Py-GC-FID (R^2 0.986, [19]), giving a R^2 of 0.975 over 14 samples (bacterial samples and purified polymers) with known amounts of PHA (Figure 4). As previously observed for the Py-GC-FID method, the relative percentage of 2-butenic acid and 2-pentenoic acid from IVT was the same relative percentage of the corresponding monomers in P(3HB-3HV) found using methanolysis.

Figure 4.

Concerning the replicability, the standard deviation of duplicates ranged from 15% to 30%, obtained by different operators during a two-year study on MMC. Therefore, a precision comparable to that of the pyrolysis-based method could be expected. The overall figures of merit had a slightly lower quality compared to the pyrolysis-based methods. Nevertheless, given the procedural advantages (ease, speed, operational

reliability) of IVT, they can be considered acceptable for the purpose of PHA monitoring in complex mixed culture system.

3.3 Titration method for determination of PHA amount.

Despite the increase in speed and simplicity of the IVT procedure, the need for a GC system, and more in general, the need for laboratory instruments can represent an important bottleneck for an extensive monitoring of MMC in bioreactors or plants. Moreover, the time needed for GC runs implies that it would be difficult to perform near-real time measurements of PHA content (in bacteria or in solutions), which is a mandatory operation for real-time monitoring. Titration is a well-established and potentially automated procedure that can be applied with low cost consumables.

For this purpose, 2-alkenoic acids (AC) produced by IVT (markers of PHA thermolysis) are the main source of weak acidity in the sample subjected to this procedure. Thus the amount of PHAs can be quantified by simple titration of AC equivalent with alkali solution.

To validate this approach, several bacterial samples (listed in Table 1) and PHA amounts were subjected to IVT and subsequently titrated to neutrality by adding a solution of NaHCO_3 . Figure 5 shows the results from titration of pure crotonic acid, IVT-treated P(3HB) and PHA-containing bacteria.

Figure 5.

As expected, the equivalents (meq) obtained by titration of crotonic acid were very close to the theoretical values (0.0116 meq/mg CA), with minor variability due to volumetric uncertainty. Titration of IVT-treated P(3HB) samples gave a linear correlation between polymer amount and meq produced; on average, 0.005 meq of acid per mg of P(3HB) was obtained. The intercept of correlation was negligible, indicating low matrix effect. Considering a yield of $0.27 \text{ g}_{\text{Crotonic acid}} \text{ g}_{\text{PHB}}^{-1}$ ($0.31 \text{ meq g}_{\text{PHB}}^{-1}$) and a significant production of dimers and trimers (contributing to acidity but to a lesser extent due to higher molecular weight), this is in agreement with the expected theoretical values.

Titration of IVT-PHA enriched bacterial samples produced 0.004 meq of acid per mg of PHA inside the bacterial cells, slightly lower than IVT-treated P(3HB) samples. This finding is in agreement with the expected production of 2-pentenoic acid from the monomer HV, characterized by a lower specific acidity ($0.010 \text{ meq/mg 2-pentenoic acid}$); and probably also by the production of a minor amount of ammonia during IVT of proteins in the sample. The correlation, although affected by a certain variability and influenced by the type of polymer (lower meq for longer AC), can be considered good enough for a preliminary screening of PHA content. This method allowed for a reliable PHA quantification in the range 10-50% on 5 mg microbial samples.

Despite the loss of information for PHA monomer composition, IVT-titration method can be directly applied in PHA producing plants without the need of special equipment. Furthermore, it can also be used when specific analytical instrumentation is not available in close proximity to bacteria production plants.

Moreover, this rationale could be applied in an automated measurement system based on the thermolysis of known amounts of freeze-dried microbial biomass and the titration of evolved compounds. This system should include a reaction chamber connected to a titration vessel equipped with a pH-meter or a conductivity probe (both should be able to detect the onset point). In terms of complexity and cost, IVT coupled with titration could be considered comparable to the actual FOS/TAC determination

4 Conclusions

Low temperature thermolysis can be used as a depolymerization method for the purpose of PHA quantification and the determination of monomeric composition. In-vial thermolysis and quantification of 2-alkenoic acids by GC-MS allows for the quantification of PHA content in freeze-dried microbial biomass, as well as for the determination of the relative amounts of different monomers in PHA copolymers. This procedure was suited to conventional laboratory apparatus, thus making it simpler than the Py-GC method previously developed but providing similar analytical performances. Direct titration of IVT products with alkaline solution allowed for the fast quantification of PHA content without any lab apparatus, except a heating plate. Although less informative than the GC method (no information on the composition of PHA), IVT coupled with the titration procedure was suitable for fieldwork and can, in principle, be used as a basis for the on-line continuous measurement of PHA content in dry bacteria or bacterial slurries.

Glossary

- Crotonic acid (CA): 2-butenic acid.

- FOS/TAC: this ratio is an indicator for assessing fermentation processes. The TAC (Totales Anorganic Carbonat) value is an estimation of the buffer capacity of the sample and the FOS (Flüchtige Organische Säuren) value corresponds to the volatile fatty acids content, calculated empirically.
- Gas Chromatography Mass Spectrometry (GC-MS).
- Long chain fatty acids (LCFA): fatty acids with aliphatic tails 13 to 21 carbons.
- In-Vial Thermolysis (IVT): high temperature treatment of bacterial biomass samples.
- Microbial Mixed Cultures (MMC): multiple strains and species of bacteria collected from a single sample.

Polyhydroxyalkanoates (PHA): linear polyesters generated by fermentation of sugars and lipids. They are generally made and used by bacteria to store energy and commercially to make bioplastics. scl-PHA: Short Chain Length Polyhydroxyalkanoate.

Acknowledgements

This work was supported by i) the University of Bologna (Ricerca Fondamentale Orientata), Regione Emilia Romagna (POR-FESR and CIPE) and Progetto Bandiera “la Fabbrica del Futuro” 2014 (Innovative and sustainable production for biopolymers-PROBIOPOL) ii) the University of Padova through the Progetto di Ateneo CPDA137517/13 titled: “Polyhydroxyalkanoates (PHAs) production from lipids containing agri-food wastes” iii) Portugal national funds through the FCT/MEC (Pest-C/EQB/LA0006/2013) and co-financed by FEDER under the PT2020 Partnership Agreement.

The Authors are grateful to Dr Rosemary Cripwell (University of Stellenbosch, South Africa) for valuable English revision and editing.

References

- [1] Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F. and Werker, A. Water Research (2015), 77, 49-63. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels.
- [2] Morgan-Sagastume, F., Karlsson, A., Johansson, P., Pratt, S., Boon, N., Lant, P., and Werker, A. Water Research (2010), 44:5196-5211. Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus.
- [3] Koller, M., Sandholzer, D., Salerno, A., Braunegg, G, Narodoslawsky, M. (2013). Biopolymer from industrial residues: Life cycle assessment of poly (hydroxyalkanoates) from whey. *Resources, conservation and recycling*, 73, 64-71, 2013.
- [4] Chanprateep, S. Journal of Bioscience and Bioengineering (2010), 110:6, 621–632. Current trends in biodegradable polyhydroxyalkanoates.
- [5] Carvalho, G., Oehmen, A., Albuquerque, M.G.E and Reis, M.A.M. New Biotechnology (2014), 31:4, 257-263. The relationship between mixed microbial culture composition and PHA production performance from fermented molasses.

- [6] Moita Fidalgo, R., Freches, A. and Lemos, P.C. Water Research (2014), 58, 9-20. Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures.
- [7] Duque, A.F., Oliveira, C.S.S, Carmo, T.D., Gouveia, A.R, Pardelha, F., Ramos, A.M and Reis, M.A.M. New Biotechnology (2014), 31:4, 276-288. Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer composition.
- [8] Povolito, S., Toffano, P., Basaglia, M. and Casella, S. Bioresource technology 101(2010), 7902–7907. Polyhydroxyalkanoates production by engineered *Cupriavidus necator* from waste material containing lactose.
- [9] Campanari, S., Silva, F.A., Bertin, L., Villano, M. and Majone, M. International Journal of Biological Macromolecules 71 (2014), 34–41. Effect of the organic loading rate on the production of polyhydroxyalkanoates in a multi-stage process aimed at the valorization of olive oil mill wastewater.
- [10] Moita Fidalgo, R. Ortigueira, J., Freches, A., Pelica, J., Goncalves, M., Mendes, B. and Lemos, P.C. New Biotechnology (2014), 31:4, 297-307. Bio-oil upgrading strategies to improve PHA production from selected aerobic mixed cultures.
- [11] Romanelli, M. G., Povolito, S., Favaro, L., Fontana, F., Basaglia, M. and Casella, S. International journal of biological macromolecules 71(2014), 21-27.. Engineering

376 *Delftia acidovorans* DSM39 to produce polyhydroxyalkanoates from slaughterhouse
 377 waste.

378

379 [12] Villano, M., Valentino, F., Barbetta, A., Martino, L., Scandola, and Majone, M.
 380 New Biotechnology (2014), 31:4, 289-296. Polyhydroxyalkanoates production with
 381 mixed microbial cultures: from culture selection to polymer recovery in a high-rate
 382 continuous process.

383

384 [13] Youwei, C.U.I., Siyuan, J.I., Pengfei, L.U., Hongyu, Z. CIESC Journal (2015),
 385 66:4, 1491-1497. Influence of F/F on PHB production by halophilic activated sludge fed
 386 by sodium acetate.

387

388 [14] Koller, M. and Rodriguez-Contreras, A. Engineering in. Life Sciences (2015), 15,
 389 558–581. Techniques for tracing PHA-producing organisms and for qualitative and
 390 quantitative analysis of intra- and extracellular PHA.

391

392 [15] Spiekermann, P., Rehm, B.H.A., Kalscheuer, R., Baumeister, D., and Steinbüchel,
 393 A. Archives of Microbiology (1999), 171:73–80. A sensitive, viable-colony staining
 394 method using Nile red for direct screening of bacteria that accumulate
 395 polyhydroxyalkanoic acids and other lipid storage compounds.

396

397 [16] Berlanga, M., Montero M.T., Borrell, J.H and Guerrero, R. International
 398 Microbiology (2006), 9:95-1022006. Rapid spectrofluorometric screening of poly-
 399 hydroxyalkanoate-producing bacteria from microbial mats.

400

401 [17] Misra, A.K., Thakur, M.S., Srinivasi, P. and Karanth, N.G. Biotechnology Letters
402 (2000), 22: 1217–1219. Screening of poly- β -hydroxybutyrate-producing
403 microorganisms using Fourier transform infrared spectroscopy.

404

405 [18] Foster, L.J.R. and Tighe, B.J. Biomaterials 16 (1995), 341-343. Enzymatic assay of
406 hydroxybutyric acid monomer formation in poly β -hydroxybutyrate) degradation
407 studies.

408

409 [19] Torri, C., Cordiani, H., Samori, C., Favaro, L. and Fabbri, D. Journal of
410 Chromatography A (2014), 1359 230–236. Fast procedure for the analysis of
411 poly(hydroxyalkanoates) in bacterial cells by off-line pyrolysis/gas-chromatography
412 with flame ionization detector.

413

414 [20] Anastassiades, M., Lehotay, S. J., Stajnbaher, D., & Schenck, F. J., Journal of
415 AOAC International (2006), 86, 412-431. Fast and easy multiresidue method employing
416 acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the
417 determination of pesticide residues in produce.

418

419 [21] Payà, P., Anastassides, M., Mack, D., Sigalova, I., Tasdelen, B., Oliva, J. and
420 Barba, A. Analytical and Bioanalytical Chemistry (2007), 389, 1697–1714. Analysis of
421 pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS)
422 pesticide multiresidue method in combination with gas and liquid chromatography and
423 tandem mass spectrometric detection.

424

425 [22] Ribeiro, C., Ribeiro, A. R., Maia, A. S., Gonçalves, V. M. F., & Tiritan, M. E.

426 Reviews in Analytical Chemistry (2014), 44, 142-185. New trends in sample

427 preparation techniques for environmental analysis.

428

429 [23] Chamkasem, N, Lee, S. and Harmon, T. Food Chemistry 192 (2016), 900–906.

430 Analysis of 19 PCB congeners in catfish tissue using a modified QuEChERS method

431 with GC–MS/MS.

432

433 [24] Tölgyessy, P., Miháliková, Z. Food Control (2016), 60, 44-49. Rapid determination

434 of total lipids in fish samples employing extraction/partitioning with acetone/ethyl

435 acetate solvent mixture and gravimetric quantification.

436

437 [25] Zhila, N., Kalacheva, G., Volova, T. Process Biochemistry (2015), 50, 69–78. Fatty

438 acid composition and polyhydroxyalkanoates production by *Cupriavidus eutrophus* B-

439 10646 cells grown on different carbon sources.

440

441 [26] Samorì, C., Basaglia, M., Casella, S., Favaro, L., Galletti, P., Giorgini, L., Marchi,

442 D., Mazzocchetti, L., Torri, C. and Tagliavini, E. Green Chemistry (2015), 17, 1047-

443 1056. Dimethyl carbonate and switchable anionic surfactants: two effective tools for the

444 extraction of polyhydroxyalkanoates from microbial biomass.

445

446 [27] G. Braunegg, B. Sonnleitner, R.M. Lafferty, European Journal of Applied
447 Microbiology and Biotechnology (1978), 6, 29–37. A rapid gas chromatographic method
448 for the determination of poly- hydroxybutyric acid in microbial biomass.
449

Figure captions

Figure 1. Sequence of the IVT steps.

1. Figure 2: Chromatograms obtained from IVT and silylation of pure P(3HB) and P(3HB-3HV). 1. (E)-trimethylsilyl but-2-enoate (2-butenic acid); 2. (E)-4-oxo-4-((trimethylsilyl)oxy)butan-2-yl but-2-enoate; 3. (2E,6E)-4-oxo-4-((trimethylsilyl)oxy)butan-2-yl 5-oxoocta-2,6-dienoate; 4. (2E,6E)-1-oxo-1-((trimethylsilyl)oxy)pentan-3-yl 7-((E)-but-2-enoyloxy)-5-oxohepta-2,6-dienoate; 5. 2E,6E)-4-oxo-4-((trimethylsilyl)oxy)butan-2-yl 7-(((E)-3-((E)-but-2-enoyloxy)acryloyl)oxy)-5-oxohepta-2,6-dienoate); 6. (E)-trimethylsilyl pent-2-enoate (2-pentenoic acid); 7. (E)-trimethylsilyl 3-(but-2-enoyloxy)pentanoate; 8. (E)-4-oxo-4-((trimethylsilyl)oxy)butan-2-yl pent-2-enoate; 9. (E)-1-oxo-1-((trimethylsilyl)oxy)pentan-3-yl pent-2-enoate

Figure 3. Chromatogram of the microbial biomass treated with IVT followed by silylation. 1. (E)-trimethylsilyl but-2-enoate (2-butenic acid), TR: 8.55 min; IS: internal standard (2-ethyl butyrate), TR: 9.77 min; 2. (E)-trimethylsilyl pent-2-enoate (2-pentenoic acid), TR: 10.32 min, analytes used for the quantitation. LCFA: long-chain fatty acids

472 Figure 4. Correlation between PHA content (% w/w of sample) determined through
473 methanolysis vs IVT.

474

475 Figure 5. Correlation between meq titrated and amount of IVT derived alkenoic acids.

476 Red line: 2-butenic acid; green line: PHB and blue line: bacterial PHA.

477

478

Figure 1

Figure 2.

Figure 3.

Figure 5.

1 **Highlights**

- 2 • In-Vial Thermolysis (IVT) allows GC-MS determination of PHA amount and
- 3 composition.
- 4 • IVT is fast, simple, and does not require special lab equipment.
- 5 • Direct titration of IVT products allows to quantify PHA without GC instrument.