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Fast procedure for the analysis of poly(hydroxyalkanoates) in bacterial cells by off-line pyrolysis/gas-chromatography with flame ionization detector

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ABSTRACT

Poly(hydroxyalkanoates) (PHAs) are polyesters formed by saturated short chain hydroxyacids, among which 3-hydroxybutanoic (HB) and 3-hydroxypentanoic (3-hydroxyvalerate, HV) are the most common monomers of homopolymers (e.g. poly(3-hydroxybutyrate), PHB) and copolymers (e.g. poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHB-HC). The most widely used approach for their determination is the polymer methanolysis followed by gas chromatography–mass spectrometry (GC–MS) analysis of the methylated monomers; this procedure generally requires the use of additional reagents (e.g. sulfuric acid) and is performed with harmful chlorinated solvents, such as chloroform. The development of fast routine solventless methods for the quantitative determination of PHAs and their monomeric composition is highly desirable to reduce sample pretreatment, speed up the analysis and decrease overall costs. It has been reported that under thermal treatment (e.g. pyrolysis, Py), PHAs are degraded in high yield (>40%, w/w_{PHA}) into the corresponding 2-alkenoic acid (e.g. crotonic acid from PHB). This work aimed at investigating this reaction for direct analysis of PHAs in bacterial cells. The sample was directly subjected to pyrolysis and trapped pyrolysis products were analyzed by GC–FID. Off-line Py/GC–FID was first optimized on pure polymers with different monomer composition (PHB, PHB-HV, PHB-HC) and then applied to bacterial samples deriving from both mixed microbial cultures or selected strains, containing various types and amounts of PHAs. The Py/GC–FID method provided RSD <15% range, limit of detection of 100 μg (1% PHAs in biomass), and results comparable to that of methanolysis ($R^2 = 0.9855$), but with minimal sample pretreatment.

1. Introduction

Poly(hydroxyalkanoates) (PHAs) are a family of biodegradable polymers that can be produced by bacteria starting from different carbon sources, such as renewable resources and waste streams. These polymers exhibit tunable physical and mechanical properties and, in principle, could substitute a portion of fossil oil derived polymers (e.g. polyethylene, PE; polypropylene, PP and polycaprolactone, PC) in the transition toward a more sustainable plastic production.

PHAs are currently produced by pure cultures of microorganisms (genetically modified bacteria or wild type strains) from glucose or other expensive substrates. However, an intrinsic high capital cost due to the process complexity (e.g., operating under sterility, necessary to maintain pure cultures) and the not negligible cost of the feedstock, which could account for up to half of the price of the PHAs production process, imply that: (i) the cost of PHAs is from 4 to 9 fold higher than that of the synthetic plastics; (ii) their applicability is restricted to a narrow range of high added value products, and (iii) the global production is limited to less than 10,000 ton y^{-1}.

Several different alternative strategies toward a more cost effective PHA production process have been scouted, including the use of mixed microbial cultures (MMC), which do not require sterility, the utilization of cheap and abundant substrates (e.g. lignocellulose, agro-industrial waste and residues) and the improvements

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of downstream processes (e.g. collection, extraction and purification steps). Concerning MMC, the need to obtain effective, stable and high quality PHAs production usually requires long bacteria acclimatization times (e.g., feast and famine regimes) which have to be monitored by huge number of systematic determinations of PHAs content in the bacterial cells. As a result, the availability of fast routine analytical methods to determine the amount and monomer PHAs composition is mandatory to reduce the costs of the research in this field.

Since PHAs are formed by esterifiable monomers, the traditional quantitative approach involves their hydrolysis or alcoholysis of the polymer into the corresponding monomer esters, analogously to the analytical procedure to determine the amount of esterifiable fatty acids in lipid samples. Differently from lipid transesterification, PHA polymers are extremely resistant to acidic hydrolysis and crotonization of the ester takes place under harsh conditions [1]. Moreover a suitable solvent, capable of dissolving the PHAs under acid conditions, as well as to dissolve the methanolysis reaction products, is required. Currently, the most common quantification method dated back to 1978 and it is based on the hydrolysis or the alcoholysis of the polymer followed by clean-up and analysis by GC–MS [2]. In this method the polymer granules and/or the lyophilized bacterial biomass are added to an excess of acidic methanol (1–4% sulfuric acid, w/v) with chloroform as co-solvent; the reaction is stirred under reflux for 4–8 h, then water is added to the mixture, the phases are separated, the organic layer is dried and subjected to analysis. Because of their excellent solvent power, chlorinated compounds are the solvents of choice for the extraction, derivatization and analytical measurements of PHAs, especially for GC analysis [3]. However, since both chloroform and dichloromethane are classified as H351, their use is undesirable from an occupational health and safety perspective, and severe restrictions are being placed for their utilization in analytical laboratories and at industrial level. As stated by Galuszka et al. [4], concerning the development of green analytical methods, research efforts should be focused on reducing sample treatment through direct analytical techniques, avoiding (i) various time-consuming derivatization steps, (ii) the generation of a large waste volume and (iii) the use of toxic reagents.

A feasible alternative to chlorinated solvents was proposed by Werker et al. [5] who reported the substitution of the chloroform/methanol mixture with other hexane/alcohols combinations; although the reaction resulted in less selective process with respect to the corresponding β-hydroxyesters, this method was effective in determining the amount and the composition of PHAs in MMC biomass. However, an intrinsic lack of "greenness" of all hydro/alcoholysis-based methods still relies on the fact that they are time consuming procedures (requiring a certain number of sample preparation steps and long reaction time) and produce high amounts of wastes, including solvents and acids.

Faster and solvent-free quantitative analysis of PHAs can be obtained by circumventing the degradation of the polymer chain into its monomers through the analysis of the whole polymer. This direct measurement can be accomplished by thermogravimetric analysis [6], infrared spectroscopy (FT-IR) [7–9], or by Nile red staining followed by fluorescence spectroscopy [10]. These approaches provide the faster way to monitor PHAs in real-time mode and are probably the most suitable techniques for process control. Their main drawback is the limited or absent information on the monomer composition of PHAs as they are not able to discriminate among hydroxybutyrate and the other hydroxyacids; moreover, since these methods are based on functional groups detection or thermal behavior, other biomolecules (e.g. lipids) can interfere with the detection of PHA analytes if applied on totally unknown substrates or on PHA-poor materials.

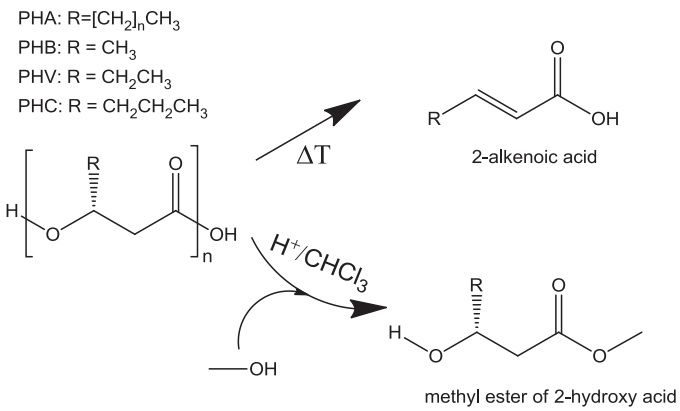

Fig. 1. PHA markers: products suitable to GC analysis obtained upon pyrolysis and methanolysis of PHAs.

An alternative yet largely unexplored strategy is the analytical pyrolysis (Py). Py of PHAs occurs at 230–330 °C [6,11] and quantitatively produces the corresponding 2-alkenoic acids and other diagnostic products [11–13], which can be analyzed on-line or trapped for subsequent GC–MS analysis [14], or by pyrolysis combined with methylation [15,16]. Moreover, it was demonstrated by bench-scale pyrolysis experiments, and the relative distribution of the pyrolysis products of PHAs is not influenced by additional components, such as salts, probably due to their high thermal stability [17,18]. Thus, it was expected that matrix effects could be limited in analytical pyrolysis of PHAs in complex samples, like bacterial biomass.

Therefore, in principle, the pyrolysis products can be used as alternative markers to identify and quantify PHA in bacterial biomass (Fig. 1). This approach was already tested for qualitative Py/GC–MS based fingerprinting in order to detect pathogens [19]. Even if the thermal behavior and reaction details are known, to the best of our knowledge, pyrolysis has never been used as an analytical tool for the direct quantification of PHAs in bacterial biomass.

In this study, we demonstrated the reliability of a novel method for the quali/quantitative determination of PHAs in bacterial biomass based on the GC–FID analysis of the pyrolysis products obtained from PHAs. In order to achieve reproducible conditions, pyrolyzes were conducted with a commercial electrically heated platinum filament pyrolyzer under off-line conditions. The main advantages of off-line over on-line pyrolysis is the possibility to add an internal standard prior to GC analysis, to couple pyrolysis with a derivatization procedure and to analyze more representative sample aliquots 10 mg [20].

2. Materials and methods

2.1. Chemicals

All standard solvents and chemicals used in this study were obtained from Sigma–Aldrich (purities ≥98%) and used without further purification. PHA pure standards, namely poly(3-hydroxybutanoate) (PHB), poly(3-hydroxybutanoate-*co*-3-hydroxyvalerate), (PHB-HV, HB/HV mass ratio of 75/25), poly(3-hydroxybutanoate-*co*-3-hydroxycaproate) (PHB-HC, HB/HC mass ratio of 90/10) were obtained from various confidential suppliers and characterized by means of standard methods (acidic methanolysis and thermogravimetric analysis) and ^{1}H NMR spectroscopy prior to use.