Non-sugar-based Bioprocess for P(3HB) Production: Combining Methanotrophs and Mealworms

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doi: https://doi.org/10.15255/CABEQ.2025.2433

Original scientific paper
Received: June 11, 2025
Accepted: September 8, 2025

The bioeconomy today relies mostly on sugar and physicochemical extraction processes, which limit both cost reduction and scale-up potential, yet these two aspects are vital for the market success of circular bioeconomy products. Gas fermentation is a platform technology that utilizes all kinds of waste biomass streams through low-cost, clean, and well-defined gaseous substrates. It can be used to obtain bioplastics. In this work, methane (CH_4) was subjected to methanotrophic conversion by *Methylocystis* sp. GB 25. The freeze-dried bacterial biomass containing 47 wt% P(3HB) – poly(3-hydroxybutyrate), was subsequently fed to mealworms. The cells were fully consumed, and the P(3HB) granules were recovered within 24 h. The final PHB purity obtained was 95 % through a simple purification step. This work demonstrated that a simple bioprocess for biopolymer extraction can be applied to small bacterial cells like methanotrophs, offering a viable alternative to classic downstream processing steps such as chlorinated solvent extraction. The methanotrophic PHB was found to exhibit high molecular weight, making it an interesting biobased, biodegradable polymer.

Keywords
sustainable feedstock, green downstream processing, extraction, bioplastics, biopolymers

Introduction

Plastics

Plastics are an abundant class of materials. While they have propelled economic development over the last 70 years, their blessing is now turning into a huge, multifaceted problem. Cheap raw materials, misguided assumptions and claims, as well as sheer convenience have created a massive industry built on linear consumption. As a matter of fact, less than 10 % of all plastics are recycled[1]. More than 98 % of today's plastics are fossil-based, and their enormous production volume, coupled with their persistence, has led to a virtual flood of waste, as well as micro- and nanoplastics (MP, NP). These are not only toxic in themselves but also act as vectors for pathogens and environmental toxins, which can be both adsorbed and absorbed, into the human body[2]. Microplastics stem not only from "obvious" sources such as spilled and leaked pellets from resin producers and their value chain, textile (polyester) fibers or tire wear, but also from paints and polymer-based coatings. Food packaging, though devoid of non-food additives, is another major source of microplastic particles consumed by humans. While polymers are large, water-insoluble molecules, they can nevertheless accumulate in the human body. There are substitutes and alternatives to plastics items, yet many modern products require the unique set of properties offered by plastics. Drinking straws serve as a prominent example. Triggered by the EU's Single-use Plastics Directive (Directive (EU) 2019/904)[3], paper straws have now replaced plastic ones. However, consumers often dislike them because paper absorbs liquid quickly, leading to a loss in mechanical integrity before the beverage is finished. A possible solution lies in bioplastics[4], which are biobased and/or biodegradable materials. All else being equal, i.e., after a one-to-one replacement of a fossil-based, non-degradable polymer with a biobased, biodegradable one – the resulting MP and NP would show a significantly shorter lifetime both in nature and in the human body, thereby limiting the duration of detrimental effects. Polymer formulations inevitably generate MP and NP, but those from non-degradable plastics are persistent. The "holy grail" of bioplastics would be a perfect "drop-in" material, offering the same processing and application properties as conventional plastics. Some such materials already exist, like thermoplastic starch (TPS) or polylactic acid (PLA), but their proliferation has been limited due to narrower processing windows, lower (thermal) stability, and higher costs compared to conventional resins. Amongst all

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bioplastics, poly(hydroxyalkanoates) (PHAs) stand out: they are highly versatile, biobased, biosynthesized, home-compostable, and biocompatible. The properties of these naturally occurring polyesters can be tuned over a wide range, making them a close-to-perfect platform for bioplastics.

PHA – the sleeping giant

While bioplastics today have a global market volume of a few million tons/year, which is roughly 2 % of all thermoplastic materials, poly(hydroxyalkanoates) PHAs account for only around 100,000 tons/year of total consumption, which is an "embryonic" scale for the polymer industry. Yet, research interest in PHA is growing, and an increasing number of manufacturers and specialized compounders are entering the market due to the high potential of PHA. Poly(3-hydroxybutyrate) (P(3HB)) is the simplest PHA. Its properties resemble those of polypropylene (PP). By producing copolymers such as poly(hydroxybutyrate-*co*-hydroxyvalerate) P(3HB-*co*-3HV), other types of PHA, such as poly(hydroxybutyrate-*co*-hydroxyhexanoate) P(3HB-*co*-3HHx) or medium chain-length PHA (mcl-PHA), as well as blends, compounds, and composites, the properties of PHA can be tuned across a very broad range. In addition, PHAs are fully biodegradable, including in marine environments, under both aerobic and anaerobic conditions. In contrast, many other bioplastics require specific conditions such as elevated temperatures to degrade[5]. Biodegradability standards such as EN 13432 set well-defined parameters, but broad, natural biodegradability, comparable to cellulose or silk, is critical for managing the end-of-life of (bio)plastic products and their MP and NP particles. At present, high production costs, stemming from sugar feedstocks and downstream processing, limit the commercial potential of PHAs. Nevertheless, this material class shows very strong potential[6]. It is estimated that up to 90 % of all fossil plastics applications could be covered by PHA[7].

Bioeconomy

It seems self-evident that we need to shift from a crude-oil-based economy to a (circular) bio-economy. Fermentation processes, compared to chemical and thermochemical processes, are more selective and can produce complex products in a one-step bioprocess. The two main hurdles in bioprocessing are downstream processing – a costly, energy-intensive step for low-concentration products, and the selection of a suitable feedstock. The classic raw material for most commercialized bioprocesses is sugar – derived from sugar beet, sugar cane, or starch (e.g., from corn, which is easily hydrolyzed). Numerous products, such as beer, wine, citric acid, lactic acid, acetic acid, acetone, butanol, and many others, are obtained via natural or genetically engineered microorganisms, yeasts, and bacteria. However, the limitations became evident with biofuels, one of the first truly high-volume bioproducts: ethanol from sugar. This first-generation (1G) biofuel triggered the "fuel vs. feed" debate, as feedstock competition raised food prices and caused undesirable effects such as land-use change. Life cycle assessments (LCA) of 1G biofuels often showed little improvement over fossil fuels. Today, an increasing number of second-generation (2G) biorefineries produce ethanol from waste biomass. However, there is still no commercialized route to low-impact bioplastics, which, like biofuels, are high-volume commodities once scaled. Thus, alternative feedstocks for bioplastics and other bulky fermentation-derived products, are urgently needed. Hydrolysis of lignocellulosic biomass to liberate sugars enzymatically is costly and produces a "cocktail" often contaminated with many other compounds. An alternative pathway via gaseous feedstocks – methane (CH_4) from biogas and syngas from biomass gasification – is highly promising offering both raw material flexibility and a clean, well-defined fermentation substrate.

PHA downstream processing

It is estimated that approximately half of PHA production costs at the commercial scale stem from downstream processing[8]. Intracellular PHA granules need to be separated from the biomass and purified without reducing molecular weight. The "classic" laboratory protocol uses chlorinated solvents such as chloroform and dichloromethane. Alternatively, "green" solvents have been tested by several researchers, as has supercritical extraction with CO_2 and a cosolvent (since CO_2 alone will only extract lipids)[9]. Commercial producers do not disclose their PHA extraction processes, but there appears to be considerable room for improvement. PHA is an intracellular energy storage compound. Recovery and purification methods include: enzymatic digestion[10,11], chemical extraction[12], and biological recovery[13–15]. However, enzymatic digestion is costly, and chemical methods are impractical at scale due to the large solvent volumes and water required to wash away the chemicals, generating wastewater that requires further treatment. One of the best solvents for PHA is chloroform; another one is dichloromethane. These chlorinated solvents are effective in laboratory trials but unsuitable for industrial application. Several articles describe the extraction of PHA with supercritical CO_2 and a cosolvent[16], with limited success to date, so improved processes are urgently needed. The key advantage of the biological extraction method is its simplicity, which allows

the recovery and purification of several hundred kilograms of PHA even in laboratory settings. Previously, mealworms (*Tenebrio molitor*) were utilized as the biological agent to perform the biological recovery of P(3HB) and P(3HB-*co*-3HHx) from dried *Cupriavidus necator* (previous names *Alcaligenes eutrophus* and *Ralstonia eutropha)* cells cultivated on plant oil and animal fats. The cells were readily consumed, and white fecal pellets containing almost 90 wt% of PHA were recovered. *C. necator* cells can accumulate up to 80 % (by mass) of PHA, and being Gram-negative, they are characterized by a comparatively thin cell wall.

In this study, we assessed the possibility of utilizing the biological recovery process to extract P(3HB) granules from *Methylocystis* sp. obtained by gas fermentation. Compared to *C. necator* cells, *Methylocystis* sp. is small and has a more rigid cell wall, warranting a study of whether the biological extraction process works. The advantage of *Methylocystis* sp. is that it can use CH_4 as its sole carbon and energy source. The strains have been tested with pure, bottled CH_4[17], natural gas[18], as well as suggested for several alternative methane sources such as landfill gas[19]. Other methanotrophs known to synthesize PHB are *Methylocystis hirsuta* CSC1[20], *Methylosinus trichosporium* OB3b[21], *Methylosinus trichosporium* IMV3011[22] and *Methylocella tundra*[23]. Methane-utilizing PHA producers are type II methanotrophs, which employ the serine pathway for C1 assimilation.

Methodology

Methanotrophic P(3PHB) production

In a 400-L loop fermenter (Fig. 1), the strain *Methylocystis* sp. GB 25[17] was continuously cultivated at 35 °C (+1 °C) in a minimal medium under sterile conditions, in a two-step process, with CH_4 as carbon and energy source. The biomass density in the fermenter was 50 g L^{-1}. The feed gas was methane: air in a 1:5 ratio, supplied at 0.05 vessel volumes per minute (VVM). After 1 day of growth, the medium was changed to be devoid of P and N for another 1.5 days. Harvesting was performed by centrifugation. The biomass (80 % water content) was freeze-dried in a laboratory-scale unit to a residual moisture content of 2 wt%. For details, see[7].

Biological recovery of P(3HB) using mealworms

Approximately 45-day-old mealworms (10.0 ± 0.2 mg), cultivated on wheat bran at 30 ± 2.0 °C, were selected for the experiment. The mealworms were starved for 24 h to clear their gastrointestinal tract from the previous feed, as described in[24]. Approximately 100 g of the mealworms were placed in a plastic tray (30 × 23 × 7 cm^3) and fed 10 g of the freeze-dried cells of *Methylocystis* sp. GB 25. The clumps of the freeze-dried cells were crushed into smaller pieces and evenly spread over the mealworms. The mealworms were left for 24 h to complete consumption of the cells in dark conditions at an ambient temperature of 30 °C and relative humidity of 60 %. After the cells had been fully consumed, the fecal pellets secreted by the mealworms were harvested by transferring the mealworms together with the frass onto a 0.50 mm screen, followed by a 0.25 mm pore-sized standard sieve, and gently shaken to separate the fecal pellets into a holding container at the bottom. The harvested fecal pellets were then dried overnight at 50 °C before characterization studies. The moisture content after drying was 1 wt%.

Purification of biologically recovered PHA

To further purify the biopolymer, the recovered fecal pellets were added to 100 mL of tap water and stirred for 30 min, at 24 °C and 200 rpm. The suspension was allowed to settle for 5 min and the water was decanted. The fecal pellets were washed continuously until almost all the water-soluble compounds and unwanted suspended solids had been removed, and the water had become clear. The fecal pellets were then scrubbed through a 0.25 mm sieve to disperse the granules, followed by stirring in water. The suspension was allowed to settle to the bottom before the excess water was discarded. To remove any remaining proteins such as phasins, the recovered fecal pellets were subjected to another washing treatment using 0.25 M NaOH solution in the ratio of 5:1 (vol/wt) and stirred for 1 h, and subsequently allowed to settle to the bottom. The NaOH solution was decanted, followed by continuous rinsing of the fecal pellet residues with tap water until the pH of the water reached 9 or lower. To brighten the polymer, 10 % (vol/vol) household bleaching solution (Clorox™, containing 5–6 wt% of sodium hypochlorite (NaOCl) in alkaline water) was added at a ratio of 5:1 (vol/wt), and the mixture was stirred for 20 minutes. The fecal pellet residue was then allowed to settle to the bottom (the density of PHB was approx. 1.25 g cm^{-3}). The bleach solution was decanted, and the fecal pellet residue was rinsed with tap water and dried overnight at 50 °C until a constant weight was achieved.

PHA characterization

Scanning electron microscopy (SEM)

SEM was used to observe the surface structure of the biologically extracted PHA granules. The purified PHA powder was viewed under a

Fig. 1 – *Pilot-scale gas fermentation unit. The horizontal part of the loop fermenter is visible. The active volume is 400 L.*

TM4000Plus II tabletop SEM (Hitachi, Japan) using an acceleration voltage of 15 kV and mixed-mode detectors at various magnifications from 100–1000×.

Gas chromatography (GC)

PHA content and composition in the cells, fecal pellet residue, and purified polymer were determined by GC analysis using a Shimadzu GC-2010 (Shimadzu, Japan) equipped with an AOC-20i Auto-Injector. Methanolysis was carried out with approximately 20 mg of freeze-dried bacterial cells or 10 mg of fecal pellet samples and chloroform-extracted PHA samples. The methanolysis solution (methanol:sulfuric acid, 85:15 v/v) was chilled at 4 °C. Then, 2 mL chloroform and 2.0 mL methanolysis solution were added into the tubes containing the freeze-dried bacterial cells and heated at 100 °C for 140 min. The tubes were then cooled down to room temperature. The methanolysis tube containing the cells was mixed with 1 mL of distilled water and vortexed (CHILTERN Auto-Vortex Mixer MT19) for 30 s to induce phase separation. The bottom chloroform layer (initial density 1.48 g cm^{-3}) was removed carefully with a Pasteur pipette and added to 0.1 g of anhydrous sodium sulfate (Na_2SO_4) in a glass vial to remove traces of water that may have been present. Next, 0.5 mL of the sample was transferred into GC vials and mixed with 0.5 mL caprylic methyl ester (CME) which served as an internal standard. The prepared sample was subjected to GC[24,25].

Differential scanning calorimetry (DSC)

The calorimetric measurement of the samples was performed using a Shimadzu DSC-60 as described previously[26]. About 3 mg of the samples were encapsulated in aluminum DSC pans and heated from − 50 °C to 200 °C (first heating scan), at a rate of 15 °C min^{-1} under a nitrogen flow of 30 mL min^{-1}. This was followed by a rapid quenching to –50 °C and subsequent reheating to 200 °C (second heating scan) at 30 mL min^{-1}. The melting temperature (T_m) and glass transition (T_g) were determined from the second heat profile.

Results and discussion

Cell consumption, fecal pellets recovery, and PHA content

The mealworms ingested the freeze-dried cells within 24 h and secreted the PHA granules in the form of brownish fecal pellets together with some mealworm exuviae, as reported previously[14,15]. In the mealworm gut, it is thought that the bacterial cells were hydrolyzed and the PHA granules released. Some of the bacterial cell biomass may have been absorbed as nutrients by the mealworms. On the other hand, the PHA granules were not hydrolyzed, probably due to the absence of suitable enzymes and/or the short retention time of the PHA granules in the mealworm gut. The PHA granules

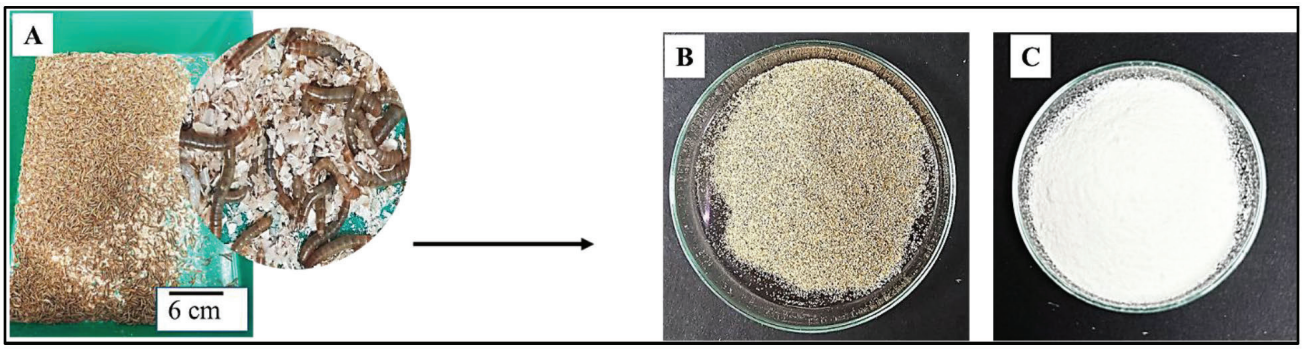

Fig. 2 – *Biological recovery of PHA using mealworms (A), fecal pellets (B) harvested from the mealworms after 24 h, and the final (C) purified PHA powder*

released from lysed cells were then compacted into pellet form in the gut and excreted as fecal pellets.

The size of an average fecal pellet was approximately 0.2 mm and consisted of numerous submicron PHA granules. The average size of a PHA granule was approximately 0.5 mm or less. Therefore, it can be estimated that a fecal pellet contained about 64 million PHA granules. The PHA granules, compacted in the form of fecal pellets, allow easy sedimentation during the washing process. Washing the fecal pellets with water removes water-soluble components and increases the purity of the PHA granules. PHA granules are known to be associated with several structural phasin proteins and catalytic proteins, such as the PHA synthase. These proteins are firmly bound to the PHA granule surface. A simple water wash does not remove such proteins. Therefore, it is necessary to use NaOH solution (0.25 M) and finally household bleach to solubilize the proteins, resulting in higher purity of the PHA granules, as shown in Fig. 2.

Further analysis of the final purified fecal pellets by gas chromatography (GC) revealed that the ultimate purity of the PHA was 95 % compared to

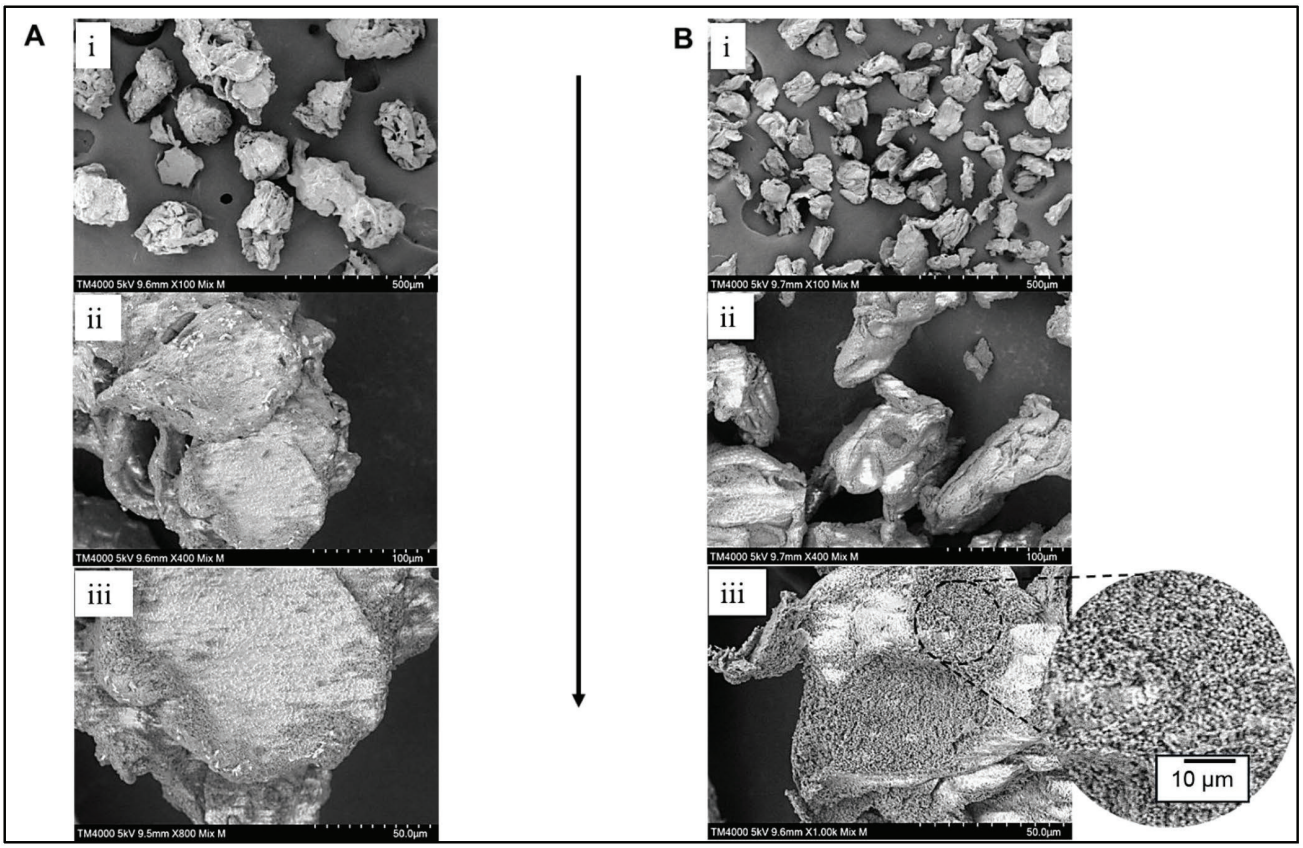

Fig. 3 – *Scanning electron micrographs showing the morphology of (A) unwashed recovered fecal pellets, and (B) washed fecal pellets at magnifications of 100–1000×. Image dimensions can be inferred from the scale bars shown at the bottom right of each micrograph.*

97 % obtained via chloroform extraction. This indicates that the biological recovery method yields a polymer of purity comparable to the reference extraction protocol with chlorinated solvents.

SEM

SEM micrographs in Fig. 3 show the appearance of the mealworm fecal pellets before (Fig. 3A i, ii, iii) and after washing and purification (Fig. 3B i, ii, iii). At higher magnification (Fig. 3B iii and the inset), the native spherical PHA granules are clearly visible. Similar morphology was reported in previous studies[14,15]). The recovery of PHA from bacterial cells using mealworms does not destroy the native PHA granular structure. Only the cellular materials surrounding the PHA granules are denatured and solubilized by the mealworm gut and the subsequent washing process. There is no evidence of the PHA granules being hydrolyzed based on previous molecular weight analysis[14].

DSC

The DSC results revealed that the melting temperature (T_m) of the unwashed and washed fecal pellets were 172 °C and 173 °C, respectively, like that of chloroform-extracted PHA (170 °C). Similar results were obtained in previous studies[13–15,27,28]. A higher melting point is generally a sign of better purity and of higher average molecular mass. However, the glass transition temperature (T_g) of biologically recovered PHA consistently shows lower values compared to solvent-extracted PHA. In this study, the T_g of chloroform-extracted P(3HB) was 3.2 °C, which was higher by 1.04 °C than that of biological recovery (2.16 °C). A similar trend was observed in all previous studies[14,29]. The reason for this is not fully understood, but it is thought to be linked to the preservation of the native granule structure during biological recovery. This can be considered a positive outcome, as it indicates better preservation of the polymer's natural state.

Bacterial cells synthesize and store high-molecular-weight PHA in an amorphous state, allowing it to function as an energy and carbon reserve. To prevent crystallization and maintain this amorphous state, it has been suggested that some plasticizer molecules are present within the PHA granules[30]. Another possible explanation is the absence of heterogeneous nucleating agents in the PHA granules. Solvent extraction destroys the native structure of PHA granules, and therefore results in crystallization of the extracted PHA. On the other hand, biological recovery preserves the native granule structure, which consists of mobile PHA molecules. Therefore, a slightly lower T_g may be observed.

Conclusion

Mealworms efficiently recovered P(3HB) granules from the bacterial cells of *Methylocystis* sp. GB 25, consistent with previous studies on other PHB-accumulating species. This indicates that neither cell type nor carbon source significantly affects the biological recovery process. Methanotrophs are low-demand, fast-growing bacteria. The biological extraction process developed here avoids the use of (chlorinated) solvents and generates mealworms and mealworm frass as byproducts. Mealworms, *Tenebrio molitor,* are increasingly valued as a sustainable, high-quality protein source for both animal feed and human nutrition, offering roughly 20 % protein by fresh weight and a balanced amino acid profile. Their coproducts could further reduce P(3HB) production costs and enhance overall carbon efficiency. Beyond feed and food applications, mealworms also yield chitin. Their ease of cultivation under controlled conditions makes them an attractive model organism for studies on insect physiology, gut microbiomes, and novel waste-to-value pathways within circular-economy frameworks. P(3HB) is the simplest PHA, and attractive copolymers such as PHBV can be obtained by adding comonomers to the fermentation broth. Through genetic engineering, other PHAs, such as medium chain-length (mcl) polymers can be made.

ACKNOWLEDGEMENTS

This work was partly funded by the European Commission under Grant Agreement no. 101112879 (project "INSPIRE"), and by the Austrian FFG under Grant Agreement no. 903872 (project "ABATE"), which is gratefully acknowledged. IZ and KS gratefully acknowledge the grant from the Ministry of Higher Education, Malaysia (203/PBIOLOGI/67811001), Science and Technology Research Partnership for Sustainable Development (SATREPS).

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