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Microbial degradation of the artificial polyhydroxyalkanoate poly(2-hydroxybutyrate-*co*-3-hydroxybutyrate) with random and block sequences using soil bacteria

Shin-ichi Hachisuka^{1,†}, Ami Iguchi^{2,†}, Hiroshi Kikukawa¹, Hiroya Tomita^{1,‡}, Toshihiko Ooi¹,
and Ken'ichiro Matsumoto^{1,*}

¹Division of Applied Chemistry, Faculty of Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan

²Graduate School of Chemical Sciences and Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan

Short running head: Microbial degradation of P(2HB-*co*-3HB)

[†]The authors contributed equally to this study.

*Corresponding author: Ken'ichiro Matsumoto

Tel.: (+81)-11-706-6610; Fax: (+81)-11-706-6610

E-mail: mken@eng.hokudai.ac.jp

[‡]Present address: International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

20 **Abstract:**

21 **Aims:** This study aimed to examine whether microorganisms capable of degrading the artificial
22 polyhydroxyalkanoate (PHA) copolymer poly(2-hydroxybutyrate-*co*-3-hydroxybutyrate)
23 [P(2HB-*co*-3HB)], with random and block sequences, are present in natural environments.
24 Previously isolated PHA-degrading soil bacteria was tested using 2HB-rich copolymers,
25 P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB), as a major carbon source. The assimilation of 2HB
26 and 3HB fractions in these copolymers and the effect of monomer sequence on degradability
27 were investigated.

28 **Methods and Results:** *Acidovorax* sp. YM1609, *Cupriavidus* sp. T1, and *Variovorax* spp. A25i
29 and A60i2 were tested in clear zone analysis to determine the depolymerization of the polymer
30 and GC analysis to assess the assimilation of the monomer component following
31 depolymerization. Strains YM1609, A25i, and A60i2 depolymerized emulsified P(2HB-*ran*-
32 3HB) and assimilated both components. For emulsified P(2HB)-*b*-P(3HB), all the strains
33 depolymerized and assimilated the 3HB component but hardly did the 2HB component within
34 two weeks.

35 **Conclusions:** This study revealed that both P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB) can be
36 degraded to some extent by soil bacteria. 2HB components were more susceptible to
37 degradation in random sequence than in block sequence; in contrast, the opposite trend was
38 rather applied for 3HB components.

39 **Impact Statement:** The results of this study suggest that the microbial degradability of 2HB-
40 containing copolymers can be regulated by their monomer sequence.

41 (198/200 words)

42

43 **Keywords:** polyhydroxyalkanoate, poly(2-hydroxybutyrate-*co*-3-hydroxybutyrate), random
44 copolymer, block copolymer, microbial degradation

45

Introduction

Plastic pollution is a global concern; for example, one study estimated that ~12,000 metric tons will accumulate in landfills and the natural environment by 2050 (Geyer et al. 2017). One approach to such an issue is to effectively use biodegradable plastics. Biodegradable polymer degradation proceeds in the following steps (Figure 1) (Lucas et al. 2008; Suzuki et al. 2021). The main chain of the polymers can be cleaved by catalytic agents, secreted microbial hydrolytic enzymes in most cases, resulting in the reduction of molecular size and the consequent generation of oligomers, dimers, and monomers. Small molecules can be taken up by microorganisms, assimilated, and mineralized through metabolism. Following all these steps is considered biodegradation.

One with relatively high biodegradability is polyhydroxyalkanoate (PHA). PHA is an aliphatic polyester produced by microorganisms; poly(3-hydroxybutyrate) [P(3HB)] is the most widely distributed in the natural environment. The biodegradability of P(3HB) and several natural PHA copolymers, such as poly(3HB-*co*-3-hydroxyvalyrate) (Doi et al. 1992; Kasuya et al. 1998; Akmal et al. 2003) and poly(3HB-*co*-3-hydroxyhexanoate) (Wang et al. 2004; Wang et al. 2018; Baidurah et al. 2019), has also been examined. Monomer units that are not naturally occurring components of PHAs are referred to as unusual monomer units [e.g., D-lactate (LA), 2-hydroxybutyrate (2HB)]. Unlike natural PHAs, artificial PHAs cannot be synthesized by natural microorganisms, and thus their biodegradability is unpredictable. For example, the

biodegradability of P(LA-*ran*-3HB) was investigated using seawater (Koh et al. 2024). In addition, P(LA-*ran*-3HB)-degrading bacteria were previously isolated from soil, which were *Variovorax* and *Acidovorax* strains (Hori et al. 2020). To our knowledge, P(LA-*ran*-3HB) is the only artificial PHA containing unusual monomer unit for which bacteria have been isolated that degrade it.

2HB is a 2-hydroxyalkanoate monomer unit with a similar structure to LA. 2HB-containing PHAs possess transparent and stretchable properties that distinguish them from naturally occurring PHAs. The properties of 2HB-containing polymers are regulated by the monomer sequence of the copolymers. In the previous study, random- and block-sequenced P(2HB-*co*-3HB) (Figure S1), P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB), respectively, exhibit distinct mechanical properties. P(2HB-*ran*-3HB) exhibited stretchability but not elasticity; in contrast, P(2HB)-*b*-P(3HB) was found to be an elastomer-like material (Kageyama et al. 2021). Despite these promising characteristics of 2HB-containing polymers, the biodegradability of 2HB-containing polymers and the effect of monomer sequences on their biodegradability have not yet been investigated.

Biological oxygen demand and/or weight loss are commonly used as indicators for polymer biodegradability, i.e., degradability in natural environments. However, these methods require enormous amounts of time and are inefficient for materials whose biodegradability is completely unknown. Therefore, as the first step, this study aimed to investigate the ability of

isolated bacteria to degrade and assimilate P(2HB-*co*-3HB) under laboratory conditions. This microbial degradability can be an indicator for estimating biodegradability in the natural environment. This study examined the microbial degradability of these polymers by artificially cultivating specific microorganisms along with the polymers; previously isolated PHA-degrading soil-derived bacteria (Sun et al. 2014; Hori et al. 2020) were prepared. Similarly, to examine the possibility of degradation first, emulsions are used in this study, which are more susceptible to degradation than materials such as films due to the larger surface area. The effect of the monomer sequence on degradability can be analyzed in detail.

Materials and Methods

Microbial resources

Bacterial strains A25i and A60i2 were previously isolated from an enrichment culture using soil derived from the campus of Hokkaido University (Sapporo, Japan) as the isolation source (Sun et al. 2014; Hori et al. 2020).

Cupriavidus sp. JCM 10169 (Hiraishi et al. 2006) and *Acidovorax* sp. JCM 10172 (Feng et al. 2004) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Research Center (Ibaraki, Japan). *Cupriavidus* sp. JCM 10169 and *Acidovorax* sp. JCM 10172 were formerly referred to as *Ralstonia pickettii* (or *Alcaligenes faecalis*) T1 and *Comamonas acidovorans* YM1609, respectively. In this study, these strains

were referred to as strains T1 and YM1609, respectively.

Culture media

Luria-Bertani (LB) and M9 media were used to culture the bacterial strains. A modified yeast extract medium (YE medium) (Sun et al. 2014) was also used with supplementation of polymer emulsion. LB medium consisted of 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ NaCl. M9 medium consisted of 12.8 g L⁻¹ Na₂HPO₄·12H₂O, 3 g L⁻¹ KH₂PO₄, 1 g L⁻¹ NH₄Cl, 0.5 g L⁻¹ NaCl, 0.1 mmol L⁻¹ CaCl₂, 2 mmol L⁻¹ MgSO₄, and 10 mg L⁻¹ thiamin·HCl. YE medium consisted of 1 g L⁻¹ (NH₄)₂SO₄, 0.25 g L⁻¹ yeast extract, 0.1 g L⁻¹ NaCl, 0.2 g L⁻¹ MgSO₄·7H₂O, 0.1 g L⁻¹ CaCl₂·2H₂O, 0.01 g L⁻¹ FeSO₄·7H₂O, and 0.8 mg L⁻¹ MnSO₄·5H₂O in 10 mmol L⁻¹ potassium phosphate (pH 7.0). Agar powder was added at 1.5 w/v% to make the medium plate. Carbon sources and antibiotics were added as necessary, details of which are described in the “Polymer preparation” and “Monomer assimilation assay” sections.

Polymer preparation

Sodium DL-3HB was purchased from Merck (Darmstadt, Germany) or Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan), whereas sodium DL-2-HB was purchased from TCI or synthesized as follows. 2-Bromobutyric acid [2BrB, 30 g, Acros Organics (Thermo Scientific Chemicals), Waltham, MA, USA] was slowly added to 1 mol L⁻¹ NaOH (250 mL) on ice. After the solution was refluxed at 120°C for 2 h using an oil bath, the conversion of all

2BrB to 2HB was confirmed by liquid chromatography (LC)-mass spectrometry. The solution was lyophilized to obtain the 2HB-Na powder. The 2HB-Na powder was dissolved in pure water and adjusted to pH 7 using 10 mol L⁻¹ NaOH.

P(3HB), P(2HB), P(2HB-*ran*-3HB), and P(2HB)-*b*-P(3HB) were biosynthesized using recombinant *Escherichia coli*. P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB) were always prepared in the same way as when the 2HB fraction was 86 and 78 mol%, respectively (Table S1). *E. coli* JM109 was transformed with pBSP_{RephaC}_{AR}pct (Kageyama et al. 2021) to produce P(3HB) and P(2HB)-*b*-P(3HB), pBSP_{RephaC}_{AR}NDFHpctalkK (Phan et al. 2023) to produce P(2HB), and pBSP_{RephaC}₁STQKpct (Kageyama et al. 2021) to produce P(2HB-*ran*-3HB). The following culture was performed at 30°C to produce P(3HB), P(2HB)-*b*-P(3HB), and P(2HB-*ran*-3HB) and 34°C to produce P(2HB) (Matsumoto and Kageyama 2019; Kageyama et al. 2021; Phan et al. 2023). Recombinant cells were cultured on LB agar plates containing 100 mg L⁻¹ ampicillin for 12 h. The seed culture was prepared using a 1.5 mL LB medium containing 100 mg L⁻¹ ampicillin for 12 h and inoculated into a 100 mL LB medium containing 2% (w/v) glucose, 100 mg L⁻¹ ampicillin, and monomer precursors. The monomer precursors were 2.5 g L⁻¹ 3HB-Na, 2.5 g L⁻¹ 2HB-Na, 4 g L⁻¹ 2HB-Na + 2 g L⁻¹ 3HB-Na, and 8.75 g L⁻¹ 2HB-Na + 1.25 g L⁻¹ 3HB-Na to produce P(3HB), P(2HB), P(2HB-*ran*-3HB), and P(2HB)-*b*-P(3HB), respectively. After culture in a shaking flask for 72 h, cells were harvested by centrifugation and lyophilized. The polymers in dried cells were extracted with chloroform at

room temperature for 72 h. The extract was filtered with a polytetrafluoroethylene membrane (0.2 μm pore size) and combined with excess hexane to precipitate the extracted polymers. The precipitant was rinsed with methanol.

The chemical structure and monomer composition of P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB) were determined using proton nuclear magnetic resonance analysis. The polymers were dissolved in CDCl_3 containing tetramethylsilane at 60°C, passed through a polytetrafluoroethylene membrane filter (0.2 μm pore size), and analyzed using an ECS-400 spectrometers (JEOL, Tokyo, Japan) (Ref.). The molecular weights of P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB) were determined using a size-exclusion chromatography system (JASCO Corp., Tokyo, Japan) equipped with two tandem Shodex K806L columns (Resonac, Tokyo, Japan). Polystyrene was used as the calibration standard. These analyses were performed on polymers obtained in the first synthesis (Table S1), and the same synthetic method was subsequently employed. The 2HB fraction was estimated by GC to be ~68 mol% at its lowest value but remained richer than the 3HB fraction.

The polymer emulsion solution was prepared as described previously (Sun et al. 2014; Hori et al. 2020) with slight modifications. The polymer (100 mg) was dissolved in CH_2Cl_2 (2 mL) by heat treatment at 60°C for 5 min. The polymer solution was mixed with water (25 mL) and emulsified by sonication on ice. CH_2Cl_2 in the emulsified solution was evaporated with stirring at 50°C for 4 h. The remaining precipitate was removed by filtration using filter paper

(No. 101; Advantec, Tokyo, Japan). The polymer emulsion solution was prepared to be $\sim 4 \text{ g L}^{-1}$, depending on the amount of evaporated water and removed precipitation. The emulsion solution was added to the medium at 25% (v/v) and adjusted to $\sim 1 \text{ g L}^{-1}$.

Degradation assay using a clear zone as an indicator

Bacterial cells were pre-cultured in LB medium using a test tube at 30°C overnight. The culture medium (1 μL) was inoculated into a YE medium plate with the emulsified polymer. The plates cultured at 30°C were observed after 3, 7, and 14 days.

Monomer assimilation assay

The pre-culture (1.5 mL) incubated in LB medium at 30°C overnight was harvested by centrifugation, rinsed with sterile water, and inoculated into M9 medium (3 mL) containing 1 g L^{-1} 3HB-Na or 2HB-Na as the sole carbon source. After 14 days of culture with shaking at 30°C, samples for analysis were prepared by filtration (0.2 μm pore size) of the supernatant resulting from centrifugation.

The 3HB and 2HB concentrations were quantified by a high-performance LC (HPLC) system with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) using 7 mmol L^{-1} H_2SO_4 as the mobile phase. The HPLC system consisted of an interface box LC-NetII/ADC (JASCO), a pump PU-4180 (JASCO), an autosampler AS-4150 (JASCO), a column oven (Shimadzu Corp., Kyoto, Japan), and a refractive index detector RI-4030 (JASCO). The flow rate was 0.8 mL min^{-1} and the column temperature was set at 60°C.

Polymer assimilation assay

The pre-culture (15 μ L) incubated in LB medium at 30°C overnight was inoculated into YE medium (1.5 mL) with emulsified polymer. After incubation at 30°C, whole cultures containing cells, remaining emulsion, and other medium components were collected and lyophilized. This operation intended to detect all 2HB and 3HB components regardless of their degree of polymerization. Uninoculated and inoculated cultures on days 7 and 14 and only uninoculated cultures on day 0 were collected. Subsequently, ethanolysis of the whole culture was performed (Hori et al. 2020). First, 500 μ L of 15% (v/v) H₂SO₄ in ethanol and 500 μ L chloroform were added to the lyophilized culture. The sample was heated at 100°C for 120 min with a 30 min interval for vortexing and cooled to room temperature. Ice-cold water (~4 mL) was added to the sample, and the sample was agitated well. The sample was centrifuged, and the lower chloroform layer was dehydrated by passing it through Na₂SO₄ and incubated with molecular sieves (13X 1/16; Fujifilm Wako Pure Chemicals) for at least 30 min. The 3HB-ethyl and 2HB-ethyl concentrations in the sample were determined using GC.

The GC system used in this study was a GC-2010 Plus (Shimadzu) equipped with a flame ionization detector, an InterCap1 column (0.25 mm I.D. $\times$ 30 m; df = 0.25 μ m; GL Sciences, Tokyo, Japan), and a hydrogen generator HG 260 (GL Sciences). Helium was used as the carrier gas; the temperature conditions were as follows: detector temperature, 310°C and injector temperature, 260°C. The column oven was initially set at 45°C for 6 min, increased to

198 90°C at 14°C min⁻¹, increased to 300°C at 250°C min⁻¹, and kept at 300°C for 5 min. 3HB-
199 ethyl and 2HB-ethyl obtained by ethanolysis of P(3HB) and P(2HB) were used to make the
200 standard curves.

201 **16S rDNA sequence analysis**

202 The 16S rDNA sequence analysis of strains A25i and A60i2 was performed. The 16S
203 rDNA fragment was amplified directly from the colonies using a primer set: 27F (5'-
204 AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and 1492R (5'-
205 GGTTACCTTGTACGACTT-3') (Turner et al. 1999). The ~1,500 bp fragments were
206 separated by agarose electrophoresis and purified using NucleoSpin Gel and PCR Clean-up
207 (Takara Bio Inc., Shiga, Japan). Sequencing analysis of the purified fragments was performed
208 using primers, 786F (5'-GATTAGATACCCTGGTAG-3') (Baker et al. 2003) and 907R (5'-
209 CCGTCAATTCMTTTRAGTTT-3') (Lane 1991). The analysis was conducted using the
210 Sanger method in Eurofins Japan (Tokyo, Japan). Sequences outside the low confidence peaks
211 were trimmed. The trimmed sequences were assembled based on the most similar sequences
212 selected by BLAST. Phylogenetic trees were constructed using the neighbor-joining method
213 by MEGA 11 (Tamura et al. 2021).

214

215 **Results**

216 **Degradation tests using polymer emulsion plates**

To evaluate the microbial degradability of 2HB-containing polymers, four soil bacterial strains were used as test subjects. Strains A25i and A60i2 were isolated from soil samples through previous studies (Sun et al. 2014; Hori et al. 2020) and had a degradation ability toward P(LA-*ran*-3HB). These P(LA-*ran*-3HB)-degrading strains were used because of the structural similarity between the 2HB and LA units. 16S rDNA sequence analysis indicated that the two strains were classified into the genus *Variovorax* (Figure 2). Strains T1 and YM1609, well-studied P(3HB)-degrading strains (Feng et al. 2004; Hiraishi et al. 2006), were chosen for comparison.

These four strains were examined for their ability to degrade emulsified polymers on YE medium plates (Figure 3). In this assay, the formation of a clear zone around the colonies indicated that insoluble polymers were depolymerized into soluble compounds such as oligomers and monomers (Figure 1). Fungal contamination was observed on the plates due to the unsterilized polymer emulsion, but the same experiment conducted repeatedly showed a similar trend (Figure S2). As expected, all strains formed clear zones on P(3HB) plates. The clear zone size did not differ much among the four strains (Table S2). In contrast, no clear zones were formed on P(2HB) plates for any strains. Regarding P(2HB-*ran*-3HB), A25i, A60i2, and YM1609 formed a clear zone, whereas T1 did not. As for P(2HB)-*b*-P(3HB), only YM1609 formed a clear zone; however, this clear zone was less transparent than those for the other polymers.

Examination of monomer assimilation abilities

This study next examined whether these four strains assimilate 2HB and 3HB, the monomer components of P(2HB-*co*-3HB). Because polymers containing 2HB units are non-natural products, bacterial strains that degrade polymers may not necessarily also metabolize their monomer components. Target strains were cultured in an M9 liquid medium with 2HB or 3HB as the sole carbon source, and the concentration of the remaining carbon source in the medium supernatant was determined (Figure 4). For all combinations of these strains and carbon sources, the carbon source was consumed based on the presence of the strains, indicating that 2HB and 3HB can be assimilated by all these strains. The 14-day incubation period was more than enough time for all these strains to consume the carbon sources.

Examination of polymer assimilation abilities

The consumption of 2HB and 3HB units in P(2HB-*co*-3HB)s by the strains was evaluated using the ethanolysis-derivatization method (Hori et al. 2020). Ethanolysis of the whole culture, including the medium and cells, converted all polymers, oligomers, and monomers into the ethyl esters of 2HB and 3HB. Therefore, decreases in 2HB and/or 3HB concentrations indicate the progress of depolymerization and subsequent assimilation of these units (Figure 1). It is crucial to confirm that the target polymer can undergo assimilation on top of depolymerization to examine the biodegradation potential of the polymer. Additionally and importantly, this method can detect the consumption of each monomer component.

Inoculated and uninoculated samples in Figure 5 were compared. For P(2HB-*ran*-3HB), strains A25i, A60i2, and YM1609 consumed 2HB and 3HB units (Figure 5A). Strain T1 hardly consumed the 2HB or 3HB unit in at least 14 days. For P(2HB)-*b*-P(3HB), none of the strains consumed 2HB in a substantially noticeable manner (Figure 5B). In contrast, all strains consumed the 3HB unit. Each unit was not completely consumed in 14 days under any of the conditions, which differed from the results of full consumption obtained when strains T1 and YM1609 were cultured with P(3HB) (Figure S3), suggesting the presence of a persistent structure in the copolymers.

Discussion

This study demonstrated that some soil bacteria can depolymerize and assimilate 2HB units in emulsified P(2HB-*ran*-3HB). P(LA-*ran*-3HB)-degrading *Variovorax* spp. degraded P(2HB-*ran*-3HB), suggesting that the degradation abilities of P(LA-*ran*-3HB) and P(2HB-*ran*-3HB) are related. In contrast, T1 hardly assimilated the 2HB units of P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB). Thus, not all P(3HB)-degrading bacteria can utilize the 2HB units of 2HB-containing polymers.

The comparison of assimilation of P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB) indicated that the P(2HB) homopolymer segment was persistent against bacterial degradation. Given that all strains assimilated monomeric 2HB, the inability to assimilate 2HB units in the block

274 copolymers was attributed to the ability of the degrading enzyme. The degradability of the 2HB
275 component might depend on the P(2HB) homopolymer chain length because the P(2HB)
276 homo-chain length in the random copolymer should be shorter than that in the block copolymer.
277 A related phenomenon was previously observed in the hydrolysis of D-LA oligomers by
278 P(3HB) depolymerases (PhaZs) (Sun et al. 2015). Hydrolysis efficiency varied with the degree
279 of polymerization of the oligomers, probably due to the aggregation conformation of the longer
280 oligomers, as predicted by molecular dynamics simulations (Hori et al. 2020). This
281 interpretation was consistent with the commonly known relationship between the molecular
282 weight of the polymers and their degradability. In general, the molecular weight, or degree of
283 polymerization, is a factor that determines the degradability of biodegradable polymers,
284 meaning that lower molecular weight polymers are more susceptible to degradation (Tokiwa
285 and Calabia 2004).

286 The result that strains A25i, A60i2, and YM1609 degraded the random copolymer,
287 whereas strain T1 did not, could also be associated with the phylogenetic tree (Figure 2). Strain
288 T1 falls into a phylogenetically distinct clade from strains A25i, A60i2, and YM1609.
289 Therefore, other strains phylogenetically close to strains A25i, A60i2, and YM1609, not only
290 *Variovorax* spp. and *Acidovorax* spp. but also *Rhodoferrax* spp., *Polaromonas* spp., *Xylophilus*
291 spp., etc., may degrade the random copolymer. These strains may have a similar degrading
292 enzyme to each other. To further clarify this, it will be necessary to isolate and analyze the

degrading enzyme(s) in the future.

Monomer assimilation tests (Figure 4) demonstrated that all strains in this study could assimilate 3HB and 2HB monomers. The metabolic pathway of 3HB, the monomer units of P(3HB), has been well studied (Kawaguchi and Doi 1992; Charles et al. 1997) (Figure S4A). 3HB is converted to acetoacetate by 3HB dehydrogenase, followed by the conversion to acetoacetyl-CoA by CoA transferase. The metabolic pathway of 2HB is also predictable based on the following studies (Figure S4B). One study reported that lactate dehydrogenase (LDH) from *Pseudomonas putida* displayed oxidase activity toward 2HB in addition to LA (Wang et al. 2014). Another study regarding 2HB production from threonine in *E. coli* revealed that 2HB production increased due to the disruption of LDH genes (Le and Park 2022). Deleting a part of the pyruvate dehydrogenase (PDH) complex reduced the degradation of 2-oxobutyrate in *E. coli* (Le and Park 2022). These studies suggested that *P. putida* and *E. coli* would use LDH and further PDH to metabolize 2HB. *Variovorax*, *Cupriavidus*, and *Acidovorax* spp. may use a similar pathway because of the wide distribution of LDH and PDH in bacteria.

This study elucidated the presence of soil bacteria that can depolymerize and assimilate emulsified P(2HB-co-3HB) copolymers containing 2HB as a primary component. As for P(2HB-ran-3HB), 2HB and 3HB components were assimilated by some bacteria. In contrast, regarding P(2HB)-b-P(3HB), the 3HB component was consumed by all strains, but the 2HB component was hardly consumed. To further investigate the causes of differences in

312 degradability by bacterial strain and monomer sequence, identifying enzyme(s) contributing to
313 degradation will be necessary. The results of this study indicate that 2HB-containing polymers
314 may be biodegradable in natural environments, although their degradation rate is probably
315 slower than that of P(3HB).

316

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318

319 **Conflict of Interest:** The authors declare no conflicts of interest.

320

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323

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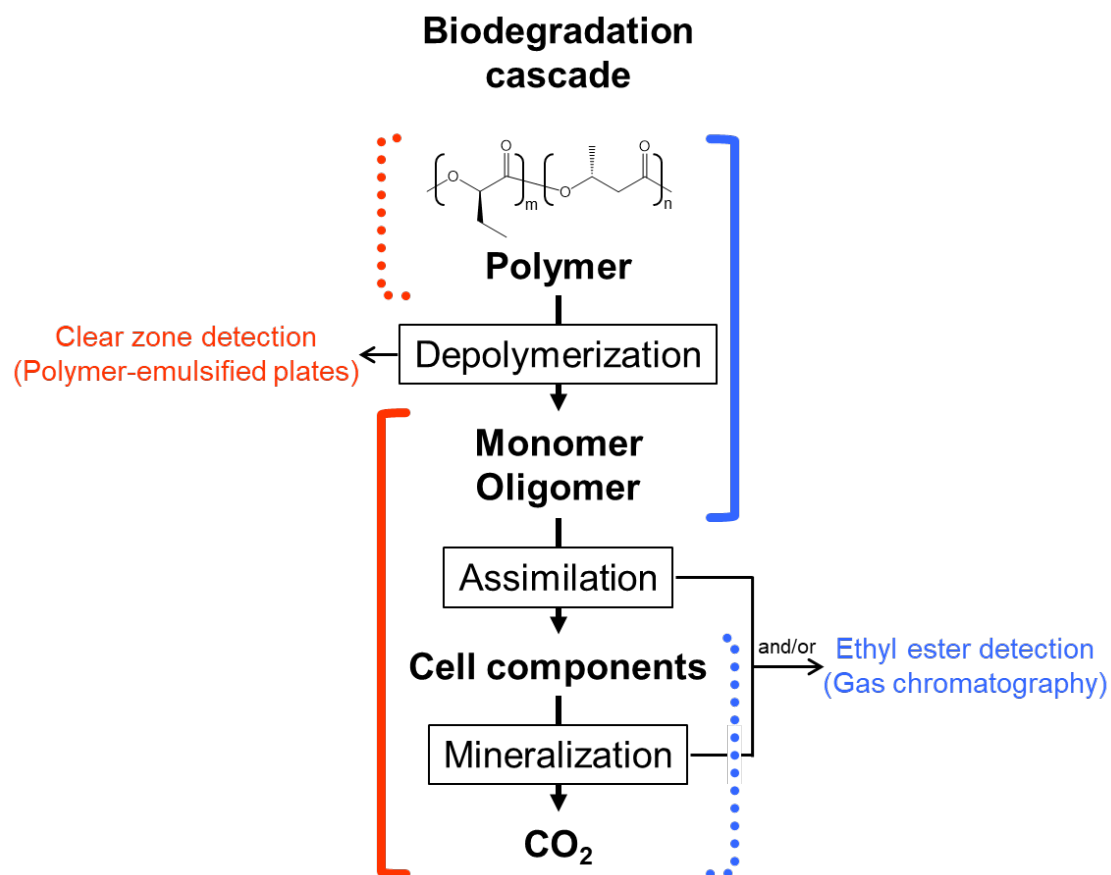
413

414 **Authors contribution statement:** S.H.: Investigation, Methodology, Writing – original draft,
415 and Writing – review & editing; A.I.: Formal Analysis and Investigation; H.K.: Methodology
416 and Writing – review & editing; H.T.: Methodology; T.O.: Methodology; K.M.:
417 Conceptualization, Funding acquisition, Methodology, Supervision, and Writing – review &
418 editing.

419

420 **Data Availability:** All data generated for this study are included in this published article and
421 the supplementary data file.

422



424

425 **Figure 1.** Conceptual diagram of biodegradation. Clear zone analysis distinguishes between
426 the two red groups, whereas gas chromatography (GC) analysis with ethanolysis derivatization
427 distinguishes between the two blue groups. The solid and dashed lines denote the targets to be
428 and not to be detected, respectively.

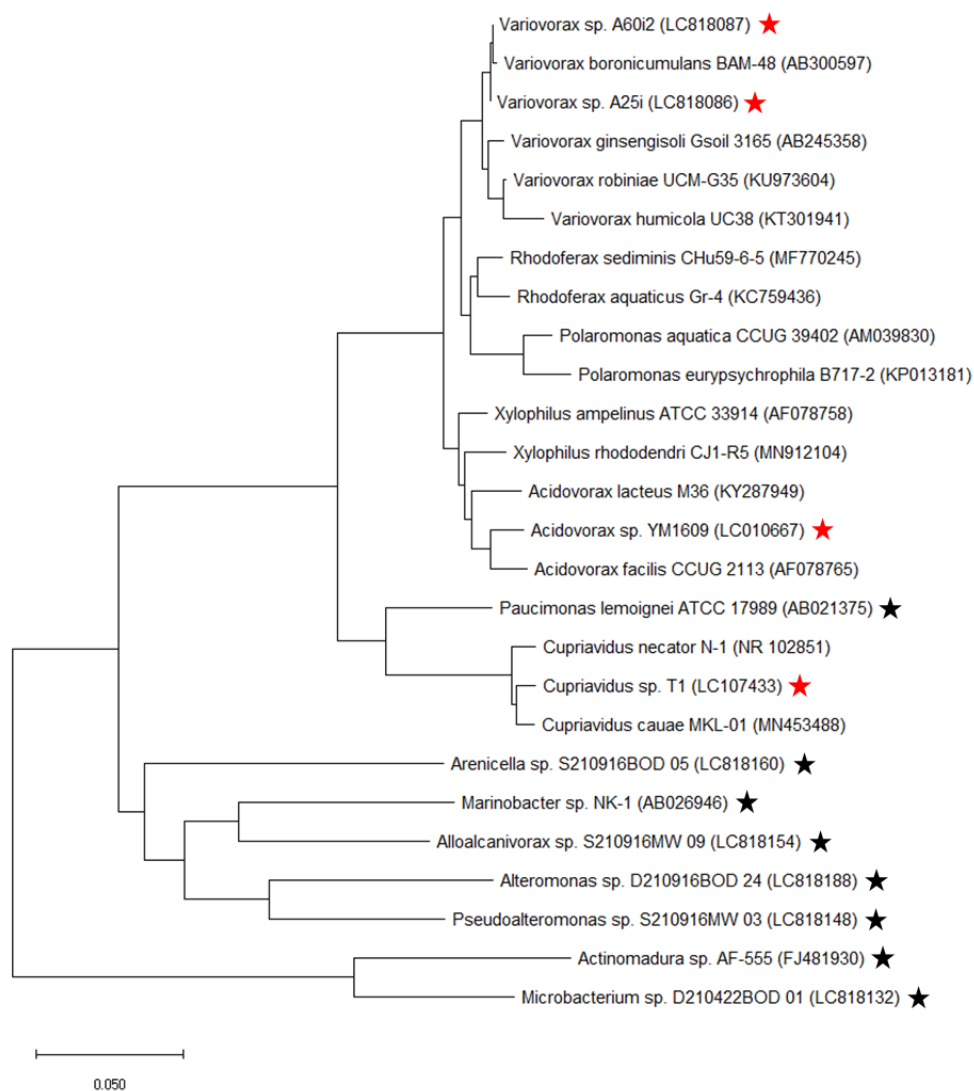


Figure 2. A phylogenetic tree of strains A25i and A60i2 and the related bacteria based on 16S rDNA sequences. Red and black stars represent the strains used in this study and the other known PHA-degrading strains (Handrick et al. 2001; Kasuya et al. 2003; Shah et al. 2010; Hachisuka et al. 2023), respectively. The lower left bar indicates a 0.5% estimated sequence divergence.

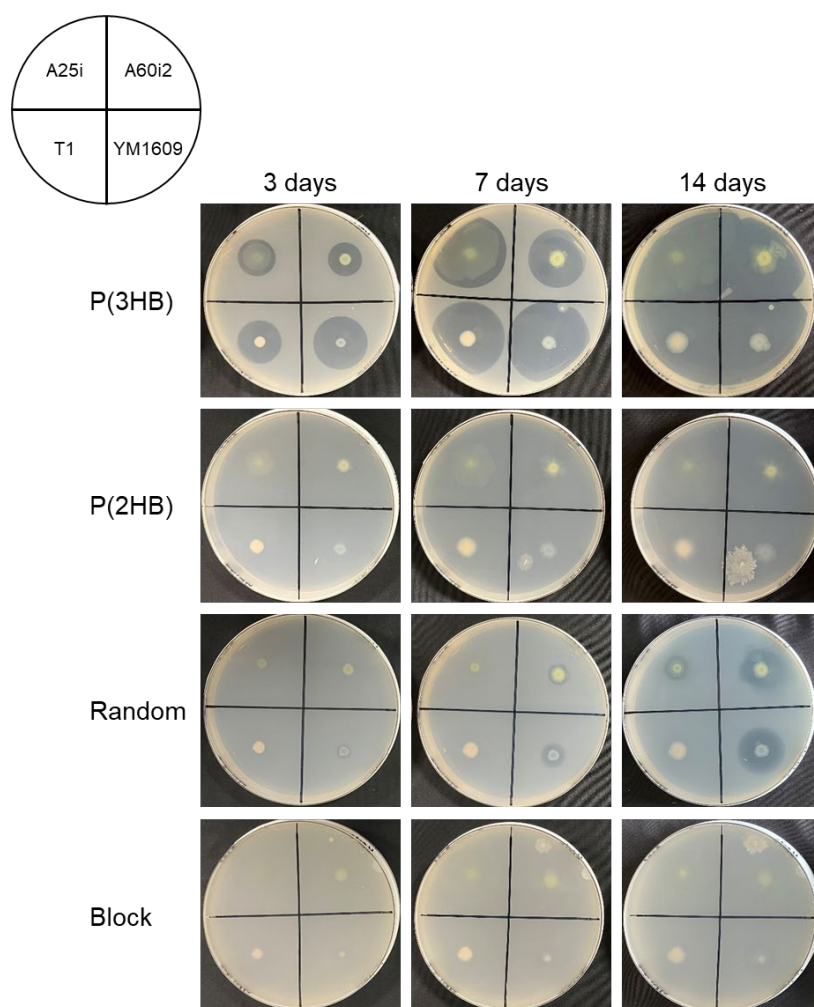


Figure 3. PHA degradation assays on medium agar plates using several PHA types. “Random” and “Block” indicate P(2HB-*ran*-3HB) and P(2HB)-*b*-P(3HB), respectively. The diameter sizes of clear zones are shown in Table S2.

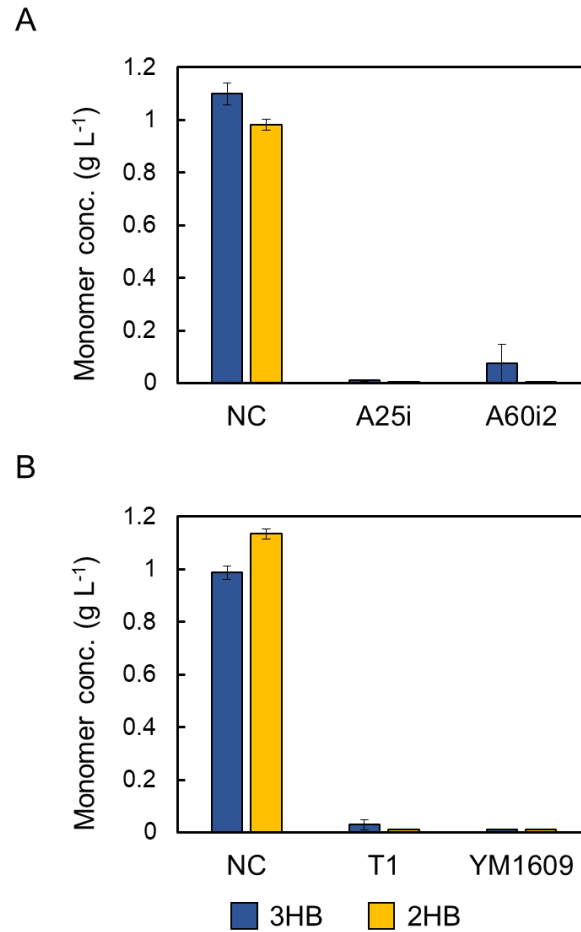
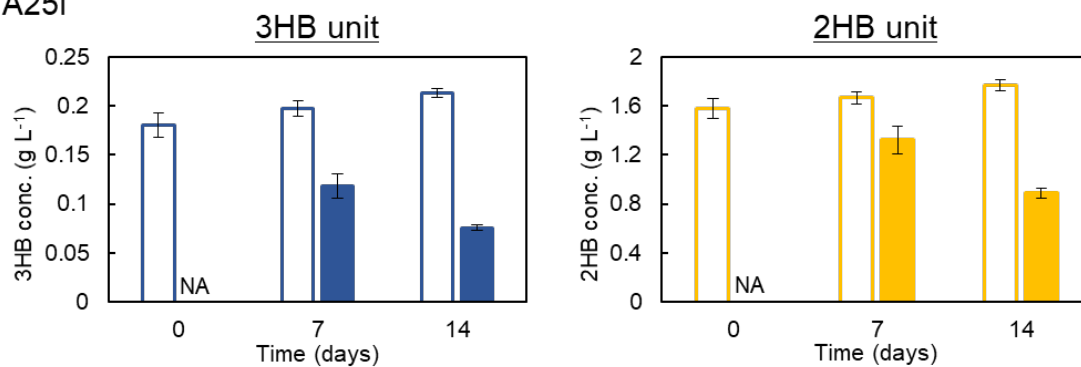


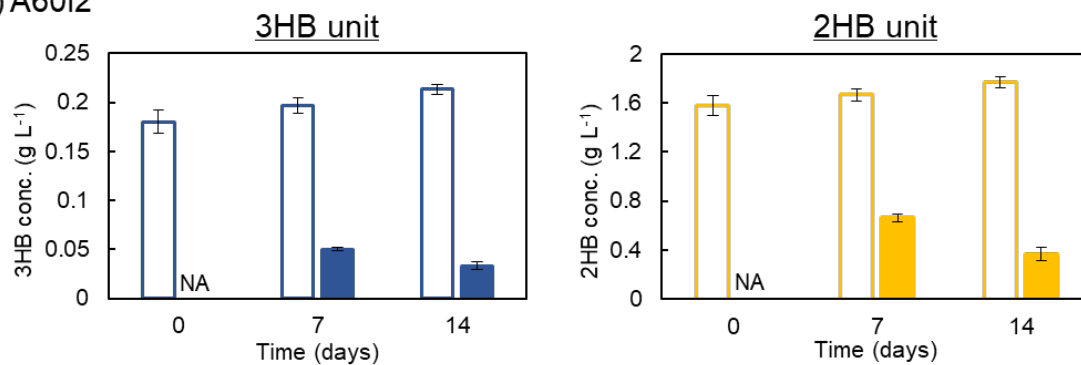
Figure 4. Monomeric 2HB and 3HB assimilation tests using A25i and A60i2 (A) and T1 and YM1609 (B). Cells were cultivated in the liquid minimum media for 14 days. Tests A and B were performed under the same conditions but not simultaneously; therefore, negative controls (NCs; no inoculation) were measured for A and B. Three independent samples for each condition were examined. Error bars represent the standard error of the mean (SEM).

A

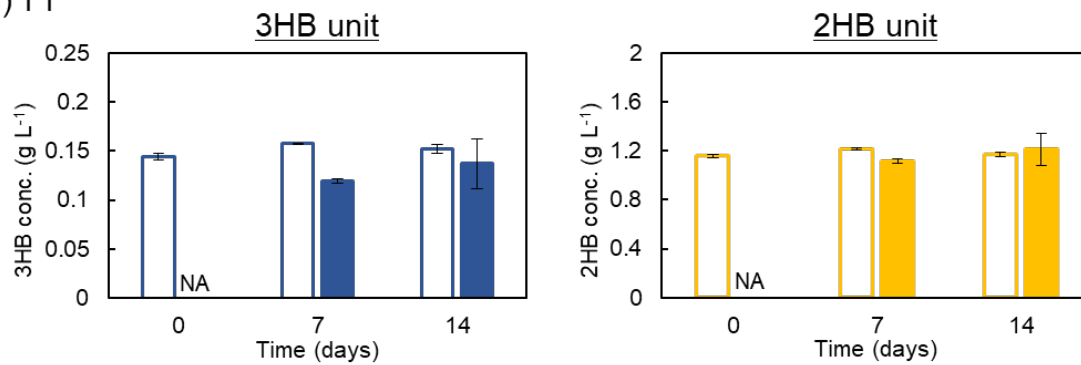
(i) A25i



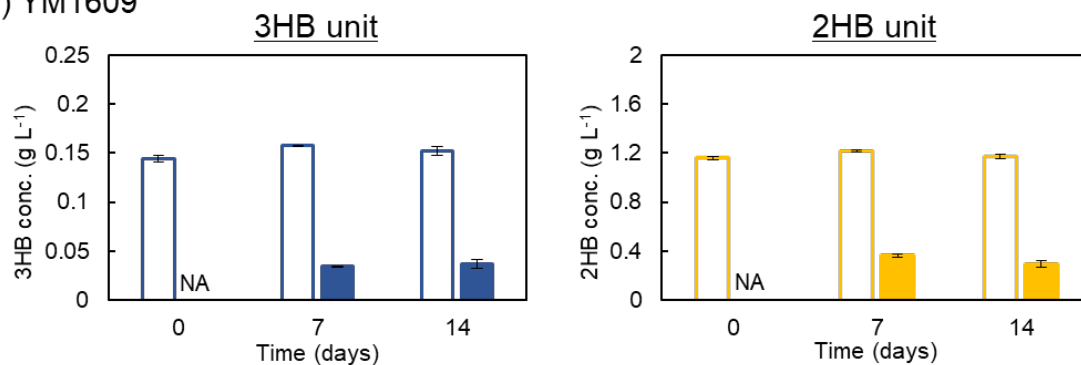
(ii) A60i2



(iii) T1

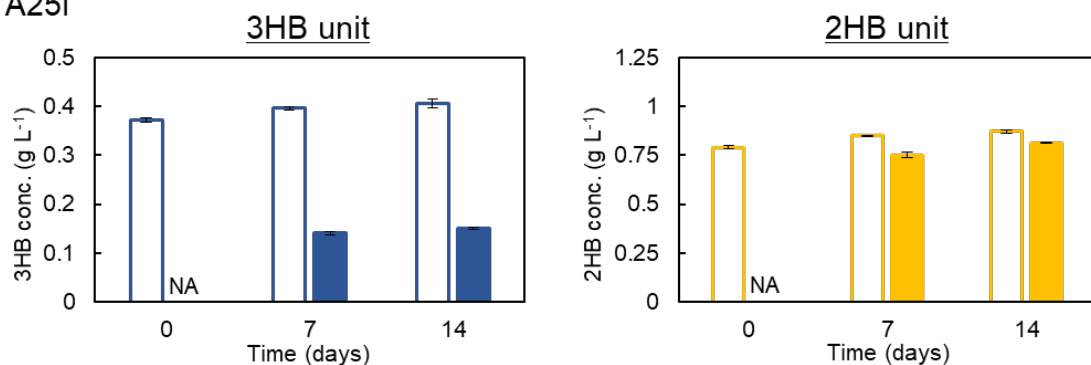


(iv) YM1609

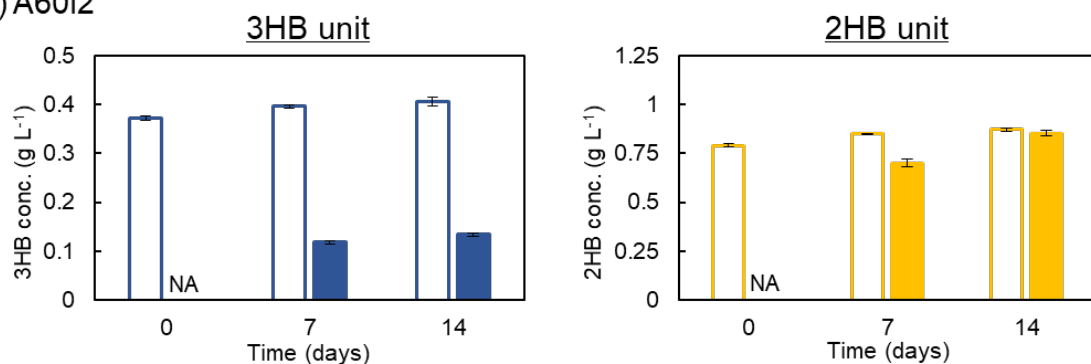


B

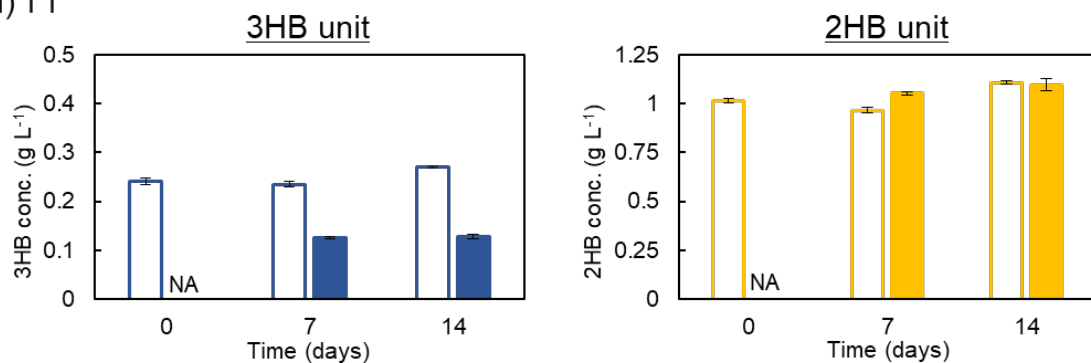
(i) A25i



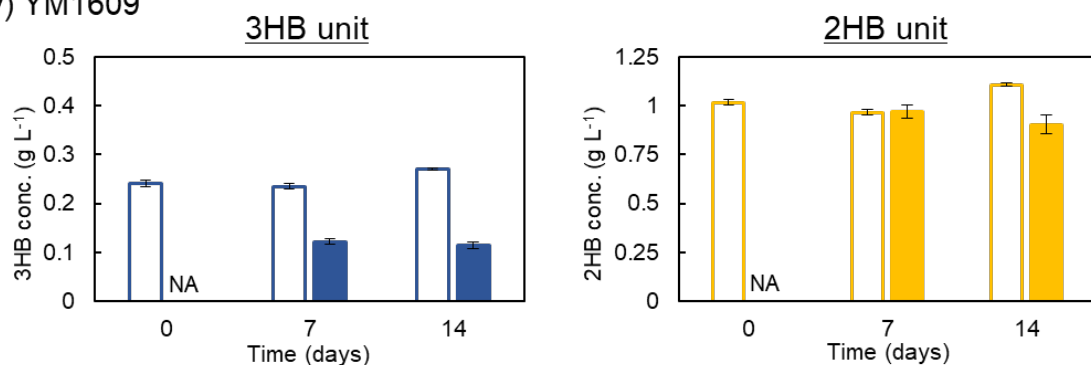
(ii) A60i2



(iii) T1



(iv) YM1609



□ 3HB (NC) ■ 3HB □ 2HB (NC) ■ 2HB

446

447 **Figure 5.** Copolymer assimilation tests using liquid media. NCs indicate uninoculated samples.

448 P(2HB-*ran*-3HB) (A) or P(2HB)-*b*-P(3HB) (B) was used as the major carbon source. Three

449 independent samples for each condition were analyzed. Error bars indicate SEM. “NA” means

450 not available.

451

452 **Supporting Information legends**

453 A separate file in a PDF document

454 Table of Contents

455 Table S1. Monomer composition and molecular weight of the copolymers used in this study.

456 Table S2. Diameter sizes of the clear zones are shown in Figure 3.

457 Figure S1. Conceptual models of random- and block-sequenced P(2HB-*co*-3HB) in this study.

458 Figure S2. An experiment under the same condition as Figure 3 performed on a different
459 occasion.

460 Figure S3. P(3HB) assimilation assays of *Cupriavidus* sp. T1 and *Acidovorax* sp. YM1609.

461 Figure S4. Metabolic pathways of 2HB and 3HB.