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Benzene containing polyhydroxyalkanoates homo- and copolymers synthesized by genome edited *Pseudomonas entomophila*

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Microbial synthesis of functional polymers has become increasingly important for industrial biotechnology. For the first time, it became possible to synthesize controllable composition of poly(3-hydroxyalkanoate) (P3HA) consisting of 3-hydroxydodecanoate (3HDD) and phenyl group on the side-chain when chromosome of *Pseudomonas entomophila* was edited to weaken its *β*-oxidation. Cultured in the presence of 5-phenylvaleric acid (PVA), the edited *P. entomophila* produced only homopolymer poly(3-hydroxy-5-phenylvalerate) or P(3HPhV). While copolyesters P(3HPhV-*co*-3HDD) of 3-hydroxy-5-phenylvalerate (3HPhV) and 3-hydroxydodecanoate (3HDD) were synthesized when the strain was grown on mixtures of PVA and dodecanoic acid (DDA). Compositions of 3HPhV in P(3HPhV-*co*-3HDD) were controllable ranging from 3% to 32% depending on DDDA/PVA ratios. Nuclear magnetic resonance (NMR) spectra clearly indicated that the polymers were homopolymer of P(3HPhV) and random copolymers of 3HPhV and 3HDD. Their mechanical and thermal properties varied dramatically depending on the monomer ratios. Our results demonstrated the possibility to produce tailor-made, novel functional PHA using the chromosome edited *P. entomophila*.

polyhydroxyalkanoates, PHB, *Pseudomonas entomophila*, *β*-oxidation, synthetic biology

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Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters synthesized by many microorganisms [1–4]. Although PHA has been developed as a type of environmentally friendly materials with low cost, it is important to introduce functionalities to PHA to improve its application [5,6]. Therefore, metabolic engineering approaches are exploited both for improving PHA production and for widening the PHA diversity [7–9]. Over 150 chiral hydroxyalkanoic acids are reported as monomers for PHA in previous investigation [5,10,11]. Generally, PHAs prepared from monomers with 3–5 (C3 to C5) carbon atoms are named as short-chain-length (SCL) PHAs, while those from monomers of C6 to C14 as medium-chain-length (MCL) PHAs [12,13]. Normally, the property of a polymer is decided by its structure [14]. Therefore, to increase the diversity of PHA structures has become a hot topic, espe-

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cially by introducing functional groups such as phenyl, halogen and unsaturated groups into PHA side chains [15–18].

Although many different organisms can utilize aromatic hydrocarbons as a carbon and energy source, only a limited number of bacteria such as *Pseudomonas putida* U, *P. putida* BM01 and *P. oleovorans*, have the ability to produce PHA-containing aromatic monomers [19–21]. *P. entomophila* L48, an entomopathogenic Gram-negative bacterium [22], shows a close relationship with the well-known MCL PHA producer *P. putida*. 70.2% of *P. entomophila* genes share orthologs in *P. putida* genome, of which >96% are found in synteny. The *P. entomophila* genome harbors most of the central catabolic genes found in *P. putida* KT2440, indicating the possibility of MCL PHA production [23]. Furthermore, *P. entomophila* was found to contain genes encoding enzymes for the catabolism of long-chain carbohydrates. Therefore, *P. entomophila* was explored as a MCL PHA producer [24]. A *P. entomophila* strain LAC23, in which its putative chromosomal β-oxidation related genes are edited (deleted) to reduce its β-oxidation ability, is able to produce MCL PHA homopolymer of 3-hydroxydodecanoate (P3HDD) when grown in the presence of dodecanoic acid (DDA) [25–27].

In this study, we aimed to investigate the possibility of synthesizing benzene-containing PHA (homo- and random copolymers) using the genome edited *P. entomophila* strain LAC23. The benzene ring is expected to add functionality to PHA, thus allowing new possible applications.

1 Materials and methods

1.1 Bacterial strains

P. entomophila LAC23, a mutant of *P. entomophila* L48, was used in this study. Its genome was edited by removing β-oxidation related genes *fadBA* and *PSEEN 0664* [22]. This mutated strain shows normal growth in the presence of DDA, and it is able to produce MCL PHA homopolymers and monomers [28–31]. Due to the weakened β-oxidation cycle, more carbon fluxes from fatty acids were directed into PHA synthesis without changing the fatty acid structures [32]. It is therefore possible that a fatty acid containing functional group(s) can be incorporated into PHA polymer chains.

1.2 Culture media and cultivation conditions

Seed cultures were incubated at 30°C in LB medium containing 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone and 10 g L^{-1} NaCl for 12 h at 200 r min^{-1} on a rotary shaker (HNY-2112B, Honor, China). They were used for a 48 h shake flask study carried out on the same rotary shaker at 200 r min^{-1} placed with 500 mL conical flasks, which contained 50 mL 4YLB medium supplemented with 12 g L^{-1} tryptone and 24 g L^{-1} yeast extract. Relevant fatty acids for PHA generation including DDA and PVA were added into the medium [33]. Figure 1 illustrates how the relevant PHA is produced via the engineered pathway.

To produce adequate amount of PHA for thermal and mechanical property characterization, we used 500 mL conical flasks containing 100 mL 4YLB medium supplemented with relevant fatty acids, and we also used more flasks.

1.3 PHA extraction and purification

Cells were harvested by centrifugation (CR21 GIII, HITACHI, Japan) at 9600 r min^{-1} for 10 min, and were directly lyophilized. Cell dry weights (CDW) were measured after lyophilization. PHA content and PHA type were

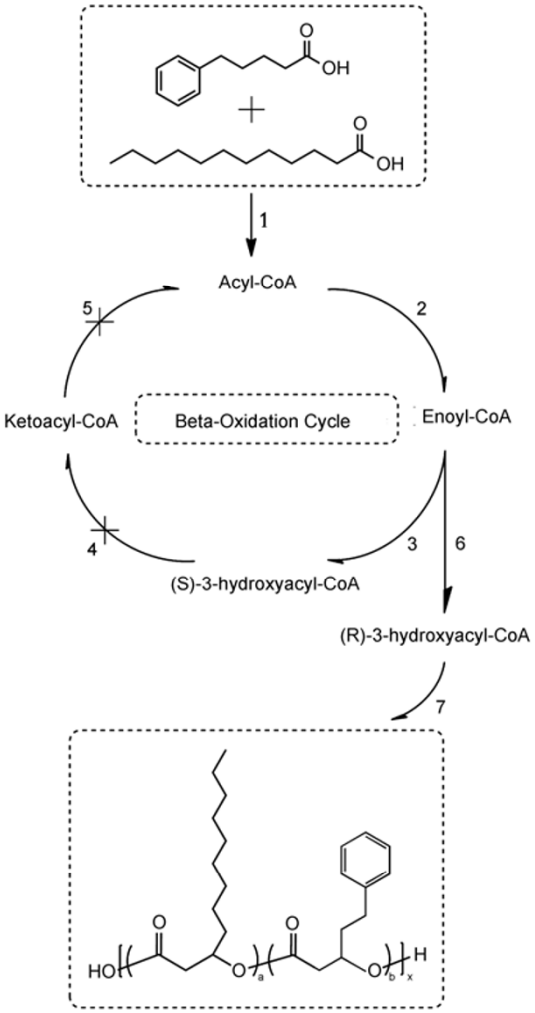


Figure 1 Major metabolic pathway for microbial synthesis of P(3HPhV-*co*-3HDD). 1: *FadD*, fatty acid CoA ligase; 2: *FadE*, acyl-coA dehydrogenase; 3: *FadBA*, S-enoyl-coA hydratase; 4: *FadB*, 3-hydroxyacyl-coA dehydrogenase; 5: *FadA,* 3-ketothiolase, *PSEEN 0664,* acetyl-coA acetyltransferase; 6: *PhaJ*, R-enoyl-coA hydratase; 7: *PhaC*, PHA synthase.

both analyzed using gas chromatography (GC-2014, SHIMADZU, Japan) after methyl esterification in chloroform [34]. The lyophilized cells were treated with chloroform at 100°C for 4 h. The intracellular PHA was obtained by Soxhlet extractor (Soxtec 2050, Foss, Denmark), and PHA was dissolved in chloroform and precipitated in an excess of 10 volumes of ethanol. The solution containing PHA precipitates was centrifuged at 8000 r min^{-1} for 10 min. After the supernatant was discarded, the purified PHA was dissolved in chloroform for film casting, and all solvents were evaporated for 7 d at room temperature to consolidate the crystallization [26].

1.4 NMR analysis of PHA

The ^{1}H and ^{13}C spectra were performed with a JEOL JNM-ECA 600 NMR spectrometer to determine the polymer composition, the chemical microstructure and the monomer sequences. Tetramethylsilane was used as the internal standard [35].

1.5 Characterization of PHA physical properties

Molecular weights were studied using gel permeation chromatography equipped with a refractive index detector (RID-10A, SHIMADZU, Japan). The measurements were carried out at 40°C with a SHIMADZU GPC-804C column. Differential scanning calorimetry data were recorded in the temperature range of –80°C to 200°C under a nitrogen flow rate of 50 mL min^{-1} on a TA instruments (DSC-Q20, TA, USA) [12]. PHA samples were casted into films by the conventional solvent-casting method for mechanical properties studies [36]. Subsequently, the PHA films were cut into dumbbell-shaped specimens with a width of 4 mm and a thickness of approximately 100 mm [36]. The stress-strain measurements of films were carried out using servo control system universal testing machine (AI-7000s, GOTECH, Taiwan, China) at room temperature.

2 Results

2.1 Production of P3HPhV homopolymer by *P. entomophila* LAC23

P. entomophila LAC23 was able to produce P(3-hydroxy-5-phenylvalerate) or P3HPhV homopolymer when PVA was added into culture medium. P3HPhV was accumulated to around 4.86wt% of the cell dry weight (Table 1). However, the P3HPhV content was very low. P3HPhV turned out to be a highly amorphous and sticky material after extraction, which limits its application. Therefore, it was necessary to enhance its rigid properties via copolymerization with another monomer. Since P(3-hydroxydodecanoate) or P3HDD produced by *P. entomophila* has strong mechanical properties [25], 3-hydroxydodecanoate (3HDD) was chosen as a copolymer monomer with 3-hydroxy-5-phenylvalerate (3HPhV) to make a mechanically useful material.

2.2 Production of P(3HPhV-*co*-3HDD) by *P. entomophila* LAC23

A mixture of PVA and DDA in cultures of the *P. entomophila* LAC23 led to the formation of copolyester P(3HPhV-*co*-3HDD) consisting of 3HPhV and 3HDD. Composition of the monomers can be adjusted by changing the ratio of DDA to PVA. For example, 17wt% P (21mol% 3HPhV-*co*-79mol% 3HDD) was accumulated when DDA/PVA ratio was 2/3, while a ratio of 0.5/3 (or 1/6) resulted in the formation of 15wt% P (63mol% 3HPhV-*co*-37mol% 3HDD) (Table 1). Interestingly, the transparency of P(3HPhV-*co*-3HDD) was dependent on monomer compositions, as increasing 3HPhV percentage in the copolymers softened the copolymer and reduced its transparency (Figure 2). The highest transparency was found to be with P(3HPhV-*co*-97mol% 3HDD).

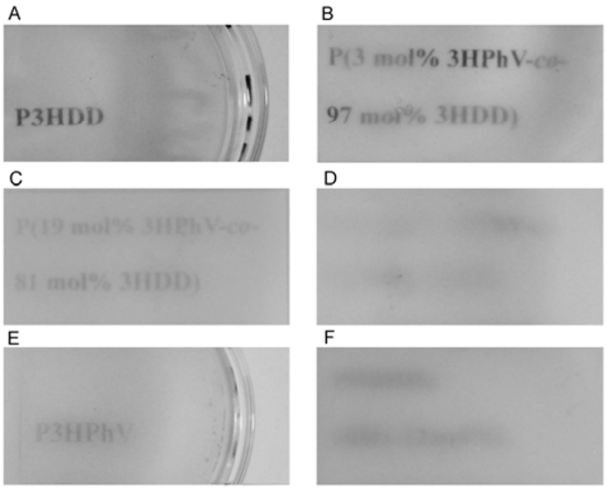
2.3 NMR microstructure analysis

The chemical structure of this novel PHA homopolymer

Table 1 P(3HPhV-*co*-3HDD) production by *P. entomophila* LAC23 grown in shake flasks containing PVA and/or DDA[a)]

PVA (g L^{-1})	DDA (g L^{-1})	CDW (g L^{-1})	PHA (g L^{-1})	PHA/CDW (wt%)	3HPhV (mol%)	3HDD (mol%)
3	0	4.34±0.31	0.21±0.06	4.86±1.13	100	0
3	0.5	4.84±0.40	0.65±0.05	13.44±0.20	63.37±0.96	36.63±0.96
3	1	5.26±0.45	0.97±0.07	18.55±0.49	39.84±0.72	60.16±0.72
3	2	5.94±0.68	1.10±0.02	17.09±0.14	21.49±0.60	78.51±0.60
3	5	5.57±0.55	1.96±0.09	35.05±2.56	1.86±0.91	98.14±0.91

a) *P. entomophila* LAC23 was cultivated in 4YLB medium supplemented with different concentrations of PVA and/or DDA for 48 h. Data shown are the averages and standard deviations of three parallel experiments. Abbreviations: PVA, 5-phenylvaleric acid; DDA, dodecanoic acid; CDW, cell dry weight. 3HPhV, 3-hydroxy-5-phenylvalerate; 3HDD, 3-hydroxydodecanoate.



Figue 2 Transparencies of P(3HPhV-*co*-3HDD) films consisting of different monomer compositions. The films were placed 1.5 cm above white papers printed with polymer names. A, P3HDD. B, P(3HPhV-*co*-97mol% 3HDD). C, P(3HPhV-*co*-81mol% 3HDD). D, P(3HPhV-*co*-68mol% 3HDD). E, P3HPhV. F, PHBHHx (3HHX: 12 mol%, control group) of 3-hydroxybutyrate and 3-hydroxyhexanoate (3HHx).

containing aromatic side chain is shown in Figure 3. The structure of homopolymer poly(3-hydroxy-5-phenylvalerate) (P(3HPhV)) was confirmed by NMR studies (Figure 4). From the ^{1}H NMR and ^{13}C NMR spectra, P(3HPhV) has the following characteristics: ^{1}H NMR ($CDCl_3$): δ=1.94 (m; 2H, H-4), 2.52–2.59 (m; 2H, H-2), 2.62 (m; 2H, H-5), 5.28 (m; 1H, H-3), 7.18 (m; 3H, H-7 and H-9), 7.28 (m; 2H, H-8) (Figure 4A); ^{13}C NMR ($CDCl_3$): δ=31.56 (C-5), 35.52 (C-4), 39.15 (C-2), 70.63 (C-3), 126.22 (C-9), 128.47 (C-7), 128.63 (C-8), 141.09 (C-6),169.43 (C-1) (Figure 4B). This result was similar to previous study [37], allowing us to conclude that the monomer of this PHA was indeed HPhV.

The microstructures of copolyester P(3HPhV-*co*-3HDD) were also studied using NMR (Figures 4 and 5). ^{1}H NMR and ^{13}C NMR spectra of P(3HPhV-*co*-3HDD) containing 32mol% 3HPhV were collected. Based on the chemical shift assignment for each proton (Figure 6A) and for carbon resonance (Figure 6B), the polymer was confirmed to be a random copolymer. Together with the well-characterized proton resonances in 3HDD units, two proton resonance peaks (PhV(7,8), PhV(9)) appeared with identical intensities which are assigned to the hydrogen on the benzene ring of 3HPhV units [37]. The expanded spectra of individual splitting resonance of each carbon in random copolymer P(3HDD-*co*-3HPhV) were clearly revealed (Figure 6B). The detailed assignment was referred to previous studies on mcl-PHA [38] and biodegradable aromatic plastics from a bacterial source [37]. All carbon resonances were split into several peaks because of the strong interaction of 3HDD and 3HPhV units in the copolymer. This phenomenon can boil down to center on triad co-monomer sequences of two

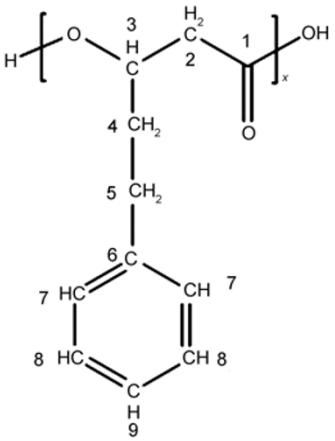


Figure 3 Chemical structure of poly(3-hydroxy-5-phenylvaleric acid) or P(3HPhV).

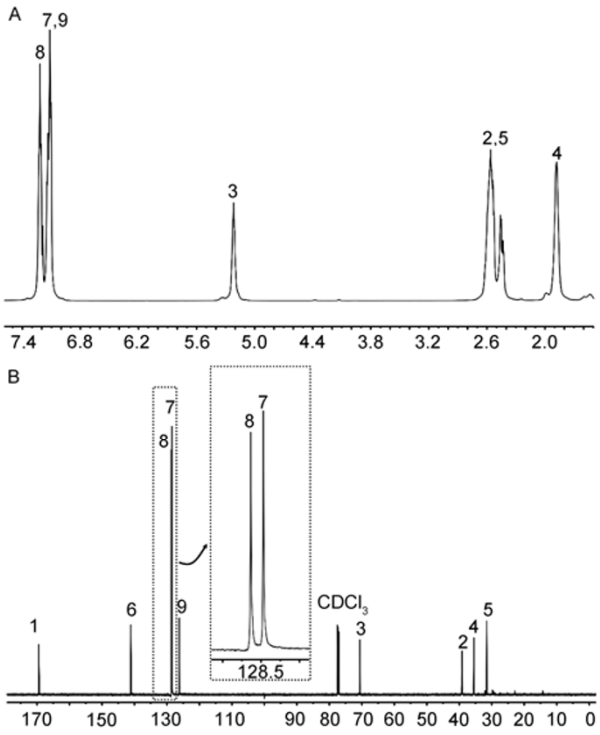


Figure 4 ^{1}H NMR (A) and ^{13}C NMR (B) spectra of homopolymer poly(3-hydroxy-5-phenylvaleric acid). Chemical shifts are in ppm and tetramethylsilane (TMS) is used as an internal chemical shift standard. Numbering scheme refers to Figure 3.

monomers 3HDD and 3HPhV, which is quite common in random PHA copolymer [3,12,39]. These analyses provided solid evidence that this PHA polymer is a random P(3HDD-*co*-3HPhV) copolymer.

2.4 Physical characterization of P(3HPhV-*co*-3HDD) copolymers

A series of P(3HPhV-*co*-3HDD) synthesized by *P. ento-*

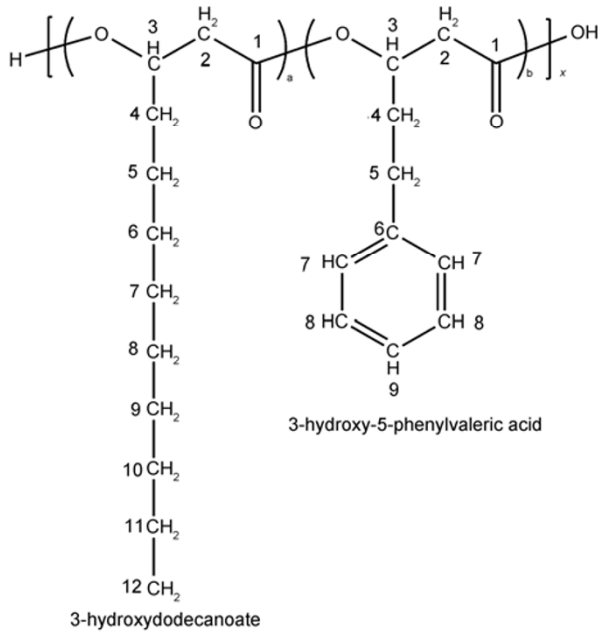


Figure 5 Chemical structure of poly(3-hydroxydodecanoate-*co*-3- hydroxy-5-phenylvaleric acid) or P(3HDD-*co*-3HPhV).

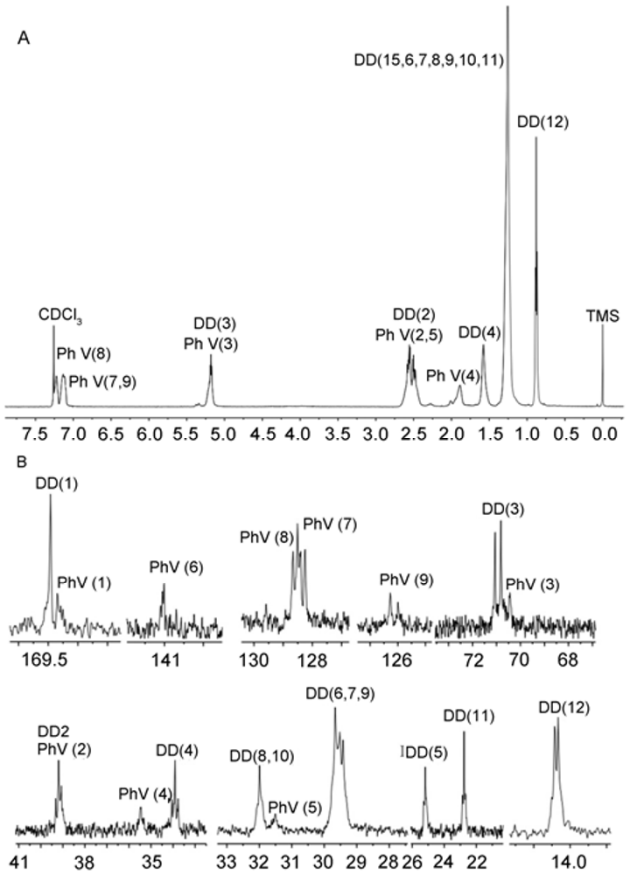


Figure 6 ^{1}H NMR (A) and ^{13}C NMR (B) spectra of random copolymer P(3HDD-*co*-3HPhV). DD and PhV refer to 3HDD and 3HPhV. The molar ratio of 3HDD and 3HPhV is 68.03% and 31.97%, respectively. Chemical shifts are in ppm and tetramethylsilane (TMS) is used as an internal chemical shift standard. Numbering scheme refers to Figure 5.

mophila LAC23 as described above (Table 1) were extracted, purified, and cut into dumbbell-shaped specimens for the following thermal and mechanical property studies. At the same time, gel permeation chromatography study showed that homopolymer P3HPhV has the lowest weight-average molecular weights (M_w) and number average-molecular weights (M_n) among all P(3HPhV-*co*-3HDD) and homopolymer P3HDD. Additionally, P3HPhV had the widest molecular weight distribution M_w/M_n, and P3HDD homopolymer had the highest M_w and M_n (Table 2).

The addition of 3HDD into P3HPhV resulted in an apparent decrease on the glass transition temperature (T_g) from 6°C to around −35°C and clearly increased the melting temperature (T_m) from 50°C to around 80°C when 3HDD ratio rose from 0mol% to 68–97mol% (Table 3). The T_m and T_g values were estimated by the result of the first and second heating scan, respectively. Therefore, the thermal property of copolyester containing phenyl group can be easily modified by changing the monomer composition, which leads to a moderate property between the two homopolymers.

P3HPhV homopolymer is a sticky material even at room temperature. Its random copolymerization with 3HDD resulted in a lower yield strength (σ_y), maximum tension strength (σ_t) and elongation at break (ε_b) than P3HDD homopolymer (Table 3). Interestingly, P(3HPhV-*co*-3HDD) with 18.70mol% 3HPhV presented a higher ε_b than P3HDD, indicating a non-linear relationship between 3HPhV content and properties. On the other hand, the Young's modulus (E) of the copolyester became higher than that of P3HDD, except P(3HPhV-*co*-3HDD) with 31.97mol% 3HPhV.

3 Discussion

Phenyl group was introduced for the first time into the PHA homopolymer P3HDD by genome edited *P. entomophila*, bringing changes to the thermal and mechanical properties of P3HDD. This strain is a mutant of *P. entomophila* L48, in which the key β-oxidation genes *fadA*, *fadB* and *PSEEN 0664* were deleted (Figure 1). Deletion of these acetyltranferase encoding genes results in weakened β-oxidation,

Table 2 Molecular weights of P3HPhV, P3HDD and P(3HPhV-*co*-3HDD)[a)]

P(3HDD-*co*-3HPhV) 3HPhV (mol%)	M_n (10^4 Da)	M_w (10^4 Da)	M_w/M_n
0	5.2	10.4	2.0
2.91	4.1	6.56	1.6
18.70	4.3	7.31	1.7
31.97	3.4	6.12	1.8
100	2.1	4.41	2.1

a) Molecular weights were studied using gel permeation chromatography. Abbreviations: M_w, weight-average molecular weight; M_n, number-average molecular weight.

Table 3 Physical characterization of the microbial P(3HPhV-*co*-3HDD)[a)]

P(3HDD-*co*-3HPhV) 3HPhV (mol%)	Thermal properties		Mechanical properties			
	T_m (°C)	T_g (°C)	σ_y (MPa)	σ_t (MPa)	ε_b (%)	E (MPa)
0	82.4	–49.3	5.5±0.8	5.5±0.9	60±34	61.1±6.4
2.91	81.00	–33.35	1.53±0.65	2.05±0.51	37.38±6.28	93.91±20.52
18.70	80.13	–35.81	3.63±0.68	4.36±0.94	86.03±39.80	94.79±34.95
31.97	75.84	–35.15	2.84±1.05	3.15±1.21	32.02±15.94	48.72±24.04
100	50.40	5.90	–	–	–	–

a) T_m, melting temperature; T_g, glass transition temperature; σ_y, yield strength; σ_t, maximum tension strength; ε_b, elongation at break; E, Young's modulus.

thus allowing more carbon fluxes being directed into PHA synthesis [25].

The successful synthesis of P(3HPhV-*co*-3HDD) indicates that genome edited *P. entomophila* is able to add functionalities to PHA. Functionalities provided by the phenyl group in the copolymer are expected to add value to PHA, as shown in this study, transparency of PHA was decreased by the increasing presence of 3HPhV in the copolymers. More novel properties are to be expected from this new material.

PHA commercialization began from early 1980s, although it has been a slow and painful process. PHA with their biodegradability and sustainability has suffered from high production cost, making it difficult to compete with petrochemical based plastics [1]. Various applications including chiral drug intermediates, biomedicines and tissue engineering, have been exploited [5], yet bureaucratic approval processes for medical usages slowed down the development. Biofuel application of PHA will also be restricted by its high cost of handling [40].

Therefore, it becomes increasingly important to add values to the expensive PHA. We think that high-value-added properties including ultra strength, shape memory, gas or liquid permeable selectivity, self healing, light wave length absorption selectivity and controllable degradation, are desirable properties for high-value-added applications. To synthesize materials with these properties, people have to introduce functional groups into the materials without being damaged during the synthesis processes.

Since most of the chemical processes including the polymerization are conducted at a high temperature, functional groups have to be protected and de-protected, which requires complete organic processing and harsh conditions. In contrast, microbial processes are carried out under gentle aqueous conditions, and functional groups survive more easily without protection. Thus, microbial processing will be a favorable approach for introducing active groups into a polymer chain, as also evidenced in this study.

However, due to the β-oxidation process of microorganisms, functional groups containing fatty acids as precursors for PHA synthesis will be mostly removed or destructed, leading to failure on functional groups incorporation into the polymer chains.

Now that β-oxidation ability in *P.* sp is weakened [25,29,30,36], fatty acids with or without functional groups can be polymerized in their original structures to become a PHA with structures precisely the same as the fatty acids we added, we are entering an era of functional PHA production with high-value-added properties.

A new functional PHA research high tide is about to come.

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