Research Article

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Production by *Rhodospirillum rubrum* Using a Two-Step Culture Strategy

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Polyhydroxyalkanoates (PHAs) are microbially synthesized biopolyesters which have attracted great attentions as a new biological material, potential alternative to traditional fossil fuel-based plastic due to their biodegradability and biocompatibility. Poly-3-hydroxybutyrate (PHB) and poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are the most common members of PHAs. In this study, the nonsulfur and facultatively phototrophic bacterium *Rhodospirillum rubrum* was cultivated to accumulate PHA by a two-step culture strategy. Gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance spectroscopy (NMR) analyses showed that PHAs synthesized from fructose was PHBV, in which the 3HV content was 46.5 mol%, which means the better mechanical property. The molecular weight, distribution, and thermal features were characterized by gel permeation chromatography (GPC), differential scanning calorimeter (DSC), and thermo gravimetric analysis (TGA), respectively. The low PDI of 1.08 revealed the narrow and evenly molar mass distribution which shows the stable features. The high melting temperature and their other physical properties implied their potential applications. The traditional process of producing PHBV involves related carbon sources such as valeric acid. However, our study clearly described a new medium formula with fructose and a complete fermentation method to produce PHBV with a high 3HV faction and low molecular distribution.

1. Introduction

Polyhydroxyalkanoates (PHAs) are a group of microbially synthesized polyesters, which are formed and accumulated by bacteria under nutrient-limiting conditions (nitrogen or phosphorus) with excess carbon source; more than 300 different microorganisms have been identified to possess this ability; these bacteria can use these intracellular PHAs as carbon and energy reserves [1, 2]. PHAs constitute for about 60–80% of the dry cell weight and can be identified by staining with Sudan Black B or using Nile blue A under fluorescent microscopy [3]. They have attracted great attentions as a new potential, biological materials alternative to traditional fossil plastic due to their good biological degradability, biocompatibility, and other special properties [4–6]. The

physicochemical, thermal, and mechanical properties of PHAs are similar to some synthetic plastics, such as polypropylene (PP), polyethylene (PE), polyethylene terephthalate (PET), and low-density polyethylene (LDPE), and together with polylactic acid (PLA), polybutylene succinate (PBS), and polypropylene carbonate (PPC), they have been considered as sustainable, environment friendly, less petroleum dependent, and the green polymers for future applications [7, 8]. Unlike the petroleum-based polymers, PHAs are completely biosynthesized and biodegradable. Therefore, PHAs has shown a more promising future in environmental concerns.

The physical properties of PHAs are determined by their molecular structure, molar masses, monomer composition, and distribution [9]. Over 150 different monomeric structures have been reported to diversify the material properties

of PHAs, ranging from rigid plastics to elastic rubbers [10, 11]. Among them, the most common and intensively studied PHAs are poly-3-hydroxybutyrate (PHB) and copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) [12]. PHB has a high-degree crystallinity, which results in its poor physical properties such as stiffness, brittleness, and low mechanical strength. Furthermore, the high melting temperature makes it difficult to be manufactured. All these inherent deficiencies hinder the homopolymer applications. However, with the incorporation of 3-hydroxyvalerate (3HV) unit into the PHB crystal structure, the crystallization process not only slowed down markedly, and melting temperature began to decrease, but also provided better tensile strength to the polymer [13–15]. Therefore, PHBV, copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), has more potential in biomedical and industrial applications than PHB. Additionally, it also demonstrates that the 3HV content plays a critical role in the physical properties of PHA polymers.

In the past decades, numerous research studies have engaged in PHAs production and many bacteria have been studied, such as *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Ralstonia*, and some photosynthetic species [16]. However, most of them can naturally synthesize polyhydroxybutyrate (PHB), but only with precursor supplying (such as valeric acid) can produce a high content of other homopolyesters or copolymers (such as PHV and PHBV) instead of PHB. For example, *Ralstonia eutropha* H16 has served as a paradigm, which stores PHB up to 90% (w/w) of its cell dry weight [17, 18], while Lindenkamp with coauthors [19] cultured *R. eutropha* H16 on valerate as a sole carbon source that accumulated almost a PHV homopolyester with 99 mol% 3HV constituents. Although more kinds of PHAs can be obtained by precursor supplying, the high cost will be another impediment to its development. Thus, a great deal of efforts should be devoted to find potential bacteria which can synthesize PHAs containing high HV fraction from unrelated carbon source. *R. rubrum*, a kind of purple nonsulfur and facultatively phototrophic bacterium, is known to produce up to 50% dry weight PHA [20]. As a PHA producer, it possesses many advantages over other bacteria. Firstly, it is one of the most versatile bacterial species in metabolism [21], which can grow in a broad range of anaerobic and aerobic conditions, autotrophically or heterotrophically, phototrophically or chemotrophically. Secondly, *R. rubrum* can produce PHAs composed of both short- and medium-chain-length monomers, while most bacteria just produce PHAs that are either short chain (C_3 to C_5) or medium chain (C_6 to C_{14}) [22]. Thirdly, it can use a variety of different carbon sources, even the toxic gas CO which is the major components of syngas [23]. Thus, *R. rubrum* has great promising to produce PHA, and its metabolic flexibility will offer all sorts of possibilities to synthesize PHBV containing a high HV fraction without the precursor supplying.

As PHA will be stored in cells under unfavorable growth conditions which indicates more time needed to get higher cell density. Hence, the two-step cultivation was preferred to accumulate PHBV by *Rhodospirillum rubrum* ATCC 11170

[24]. Firstly, the bacteria were cultured in growth medium so that a high cell density was achieved in a short time. Then, the cell was centrifuged and suspended in a mineral medium to synthesize PHBV. In the later medium, fructose is the sole carbon source and NH_4Cl is the restricted nitrogen source.

2. Materials and Methods

2.1. Strain and Medium. *R. rubrum* (ATCC 11170) strain used in this study was obtained from the American Type Culture Collection (ATCC), cultured in an enriched broth medium (30 g of Trypticase soy broth per liter water) at 26°C and 170 rpm aerobically in dark for 4 days, and the bacterium solution that started out colorless and transparent became red and opaque over time. The PHA production was assessed in *R. rubrum* cultured in fermentation medium containing excessive carbon source and deficient nitrogen source. In these experiments, the broth medium culture was collected by centrifugation at 4000 rpm for 15 min at 4°C, and then the cells were washed once with fermentation medium and resuspended in it. Cultures were shaken at 26°C and 170 rpm in dark, but no strictly anaerobic growth.

A mineral medium (MM) was also used in this study, the composition of which medium was as follows (per liter of distilled water): 5.23 g $NaH_2PO_4 \cdot 2H_2O$, 11.55 g $Na_2HPO_4 \cdot 12H_2O$, 0.31 g K_2SO_4 , 0.04 g NaOH, 0.1 g NH_4Cl , 0.79 g $MgSO_4 \cdot 7H_2O$, 0.061 g $CaCl_2 \cdot 2H_2O$, and 1 mL trace metal solution (0.048 g $CuSO_4 \cdot 5H_2O$, 0.24 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $MnSO_4$ and 1.5 g $FeSO_4 \cdot 7H_2O$ per 100 mL), maintained pH around 7.0. It is worth noting that NH_4Cl , trace metal solution, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ should be sterilized alone, and trace metal solution should be prepared when it will be used. Then add 10 g fructose as carbon source, the nitrogen source was NH_4Cl , and the carbon nitrogen ratio (C/N) was 154:1.

2.2. PHA Extraction and Purification. Cells were harvested from liquid cultures by centrifugation at 4000 rpm for 15 min, and the cell pellets were lyophilized and stored at $-80^\circ C$ until analysis.

Dried cells were treated with hot chloroform solution at 100°C for at least 4 h. The ratio of chloroform solution to dried cells was 15:1 (v/w). After cooling to room temperature, equal volume of water was added and mixed evenly. After centrifugation at 5000 rpm for 5 min, filter the organic phase by filtration in order to remove of cell debris and obtain clarified the organic phase, followed by precipitation with 10 volume of prechilled ethanol. Precipitation was collected by centrifugation at 5000 rpm for 30 min. To obtain a pure product, the precipitate was redissolved in chloroform and the process was repeated. The collected PHA was dried in an oven to remove all residual solvent [25].

2.3. GC and GC-MS Analysis. PHAs concentration and composition were determined using 80 mg dry cell samples and analyzed by gas chromatography (GC). At first, dried cells were subjected to derivatization in a mixture of 2 mL esterified liquid (1 g/L sodium benzoate was used as an

internal standard and dissolved in 97% (v/v) methanol and 3% (v/v) concentrated sulfuric acid) and 2 mL chloroform solution at 100°C for 4 h. Then, the resulting sample (1 μ L) methanolyzed was assayed using GC Clarus 580 (PerkinElmer, USA) equipped with a BD-FFAP column (30 m $\times$ 0.32 mm $\times$ 0.25 μ m) [26]. Nitrogen was separation carrier gas (2 mL/min). The oven temperature was programmed at an initial temperature of 80°C for 1.5 min and subsequently raised at 30°C/min to 140°C and 40°C/min to 220°C and held for 0.5 min. The temperatures of the injector and detector were 200 and 220°C, respectively [27].

The purified PHAs sample was prepared with the same derivatization method as mentioned above, but water was used to remove sulfuric acid. A 1 μ L portion of methyl esters was injected into a GC-MS (Agilent 7890B/7693, HP-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m)) to determine the composition of the produced polymer [28]. The temperature of the injection port, interface, quadrupole, and ion source was set at 250, 280, 130, and 250°C, respectively. The temperature gradient was programmed as described by Tan and coauthors [29].

2.4. NMR Analysis. ^1H -NMR analysis is an important means to detect the chemical structure of PHAs. The monomer compositions of PHAs copolymers were investigated using a Bruker AVANCE III 400 MHz NMR spectrometer (Bruker, Germany). The detailed procedure was according to that mentioned by Han and coauthors [30], which is described as follows: 10 mg purified PHA was dissolved in 1 mL deuterated chloroform or just at a concentration of 10 mg/mL. Tetramethylsilane (TMS) was used as the internal standard, and the 400 MHz ^1H -NMR spectra were recorded at 25°C.

2.5. Determination of the Molecular Weight. Gel permeation chromatography (GPC) analyses were carried out using a pump (Shimadzu LC-10AD VP), detector (Shimadzu RID-10A), and Shodex GPC Chirfm 4×10^5 and 4×10^6 Mixed Bed column (300 $\times$ 8 mm). It can estimate the average molecular weight and molecular weight distribution of PHAs. Analyses were performed at 35°C using chloroform as a mobile phase at a flow rate of 1 mL/min. The instrument was calibrated with polystyrene narrow standards and then constructed a calibration curve [31].

2.6. Measurement of Thermodynamic Properties. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) are the two most common methods. DSC and TGA were performed with DTG-60A Instrument (Shimadzu, Japan), and the scanning temperature was from 50°C to 500°C. The empirical method for DSC refers to Cibichakravarthy et al. [32]. The cool crystallization temperature (T_c) and melting temperature (T_m) were taken at summit of crystal peak and summit of melting peak in the second heating run, respectively.

The thermal cyclization and the thermal stabilities of PHA were analyzed by TGA, and the detail processes are as follows [33]: 14.6712 mg PHAs samples were heated at

10°C/min from 50°C to 500°C in a nitrogen atmosphere at a flow rate of 50 mL/min.

3. Results and Discussion

3.1. PHA Accumulation and Preliminary Identification. *R. rubrum* was cultured in enriched broth medium and mineral medium (MM) by the two-step method described above. PHAs were extracted and detected according to the method described above. 3-hydroxybutyrate acid and 3-hydroxypentanoic acid were used as positive controls. The values of the peak areas were determined according to their retention time. According to the retention time, it can be preliminarily determined that the PHAs accumulated by *R. rubrum* in this study contained 3HB and 3HV, which indicated that PHBV was accumulated successfully by *R. rubrum*.

3.2. Structure Analysis of the PHA. To further confirm the structure of the PHAs, GC-MS, and NMR were used to determine the structure of the PHBV. We used water and chloroform to extract and purify PHA, and then methyl ester of purified PHAs was prepared for GC-MS analysis to determine the monomer composition of it. Two chromatogram peaks with a retention time of 4.201 and 5.636 min were compared with the data from the mass spectrum library. The contrast results showed that the two peaks matched that of methyl esters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate 3HV (Figure 1). This can be concluded that the material was PHAs; moreover, it was composed of 3HB and 3HV monomers, and the species of PHAs was PHBV.

In the process of water extraction, 3HB methyl ester and 3HV methyl ester were damaged to varying degrees. Therefore, the GC-MS method cannot accurately determine the monomer ratio of 3HB and 3HV, so we use the NMR hydrogen spectrum scanning to further determine it. In the spectrum, each peak corresponds to a certain carbon atom in monomers, and two proton peaks of the CH group at 5.10–5.20 and 5.21–5.30 ppm belonged to 3HV and 3HB, respectively (Figure 2). The result of ^1H -NMR analysis showed that the copolymers were PHBV, which is in full agreement with the results of GC-MS analysis. The peak area of 3HB was set as the standard of 1.0, and the ratio of the area of 3HV was 0.87. The content of 3HV can be calculated as follows: 3HV content (mol%) = area of V_3 / (area of B_3 + area of V_3) $\times$ 100%. It was calculated that the mole fraction of 3HV was 46.5%. Taking on Zhao et al. [28], they found a novel PHBV-accumulating species *Halogramma amylolyticum* TNN58 synthesizing a PHBV containing 20 mol% 3HV fraction. Obviously, our result was significant in producing PHBV with high HV content with no genetic/metabolic engineering or precursor supplying so far, which is the highest 3HV content in PHBV accumulated by *R. rubrum*.

The two-step culture method has been implemented in previous publications to produce PHA by *R. rubrum*, and the RRNCO medium was used in those studies [34]. The main

FIGURE 1: Composition analysis of purified PHA-extracted material from *R. rubrum* (ATCC 11170) by GC-MS.

carbon source in the RRNCO medium was sodium acetate, the nitrogen source was yeast extract and NH_4Cl , and the carbon source was in small amount. The cultures were shaken under 5000 lx light intensity and strictly anaerobic conditions. As a result, PHBV in which the 3HV content was only 0.7% was obtained [20]. In contrast, in the study, fructose was used as the sole carbon source, the carbon

nitrogen ratio (C/N) was 154 : 1, and the 3HV content was up to 46.5%. Therefore, the carbon sources and the C/N ratio may be important factors affecting the content of 3HV. The higher the C/N ratio, the more unbalanced the growth environment, which is more beneficial for the accumulation of PHBV with high HV content. Furthermore, the constituent of RRNCO was more complex. Biotin, chelated iron-

FIGURE 2: The monomer compositions analysis of purified PHA-extracted material from *R. rubrum* (ATCC 11170) by ^1H -NMR.

FIGURE 3: TGA and DSC thermographs of purified PHBV extracted material from *R. rubrum* (ATCC 11170).

molybdenum solution, NiCl_2 , and MOPS in RRNCO were not needed in the medium, and the common inorganic salt solution was enough. Additionally, it is not necessary to have light and to be strictly anaerobic. All of these showed that the method cannot only produce high content of 3HV, but also save energy and operate conveniently.

3.3. Physical Properties of PHBV. The physical properties of polymers are characterized by thermodynamic parameters, molecular weight distribution, and mechanical properties. In this study, the properties of PHBV were investigated by TGA and DSC analyses described above (Figure 3) and are summarized in Table 1. Among all the

TABLE 1: Thermal properties of PHBV.

Sample	Thermal properties		
	T_c^a ($^{\circ}\text{C}$)	T_m^a ($^{\circ}\text{C}$)	T_d^b ($^{\circ}\text{C}$)
PHBV	96.15	173.56	279.24

^aDetermined from the DSC second heating run. ^bDetermined from the TGA at the beginning of 5% degradation.

FIGURE 4: (a) Standard curve of molecular weight of polystyrene; (b) Standard curve of number-average molar mass (M_n) of polystyrene; (c) Standard curve of weight-average molar mass (M_w) of polystyrene.

thermal properties, the thermal stability is particularly important. PHAs with low melting temperatures and/or increased thermal stability are desirable. The thermal stability of the material was evaluated and measured by the degradation temperature (T_d) corresponding to the mass loss of 5%. As shown in Figure 4, the T_d , T_c , and T_m of the PHBV in this study was 279.236 $^{\circ}\text{C}$, 96.150 $^{\circ}\text{C}$, and 173.560 $^{\circ}\text{C}$, respectively. As reported, PHB was thermally unstable and possibly degradable when submitted to usual process conditions. Its T_d was about 220 $^{\circ}\text{C}$ which was near its melting point (about 180 $^{\circ}\text{C}$) [35, 36]. Compared with PHB, the PHBV obtained in the study had a higher T_d (279.236 $^{\circ}\text{C}$), which was far apart from its melting temperature T_m . This showed that it had better thermal stability and was not easy to be degraded in the process of processing. Also, the lower T_m implied the possibility of being processed at a lower temperature. This material had better prospect applications. And, the

T_c was a little higher than other reported materials. The higher T_c meant it needed more time to be crystallized completely. According to this point, the crystallinity percentages of our PHBV might be a little higher, and this enhanced the strength of our material, as the crystallization of PHB was not easily disturbed by 3HV monomers. Therefore, the reason of the high crystallinity percentages might be some short segments of PHB in this polymer.

GPC is widely used to measure the average molecular weight and molar mass distribution of PHBV. In this research, it was mixed with the polystyrene with molecular weights of 20 K, 50 K, 100 K, 200 K, 600 K, and 900 K. The retention time was measured by GPC, and the standard curve was obtained (Figure 4) (retention time as the abscissa, $\log_{10} M$, $\log_{10} M_n$, $\log_{10} M_w$ as the ordinate). Then, we measured our PHBV sample and obtained its retention time (12.4625 min). The molecular weight, the

TABLE 2: Molar masses and mechanical properties of PHBV polymers.

Sample	M (10^5)	M_n (10^5)	M_w (10^5)	PDI (M_w/M_n)
PHBV	5.13	4.93	5.31	1.08

number-average molar mass (M_n), and the weight-average molar mass (M_w) were calculated and filled in Table 2. The low PDI of 1.08 reflected that the molar mass distribution of our material was narrow and even. The result was lower than the PDI of PHBV biosynthesized by other known microorganisms [37–39].

4. Conclusions

In this study, fructose was the sole carbon source in the fermentation medium for PHBV accumulation, and experiments were performed by a two-step batch strategy. The results showed that the PHA accumulated in the study was PHBV, which consisted of some short segments of PHB and PHV linked together. The content of 3HV in the PHBV was 46.5%, which was much higher than that mentioned in previous reports. The physical properties such as molar masses and thermal properties of the PHBV were tested. The PDI was lower than those biosynthesized by other known microorganisms, which means the molar mass distribution of our material was narrow and even. The T_d and T_m were 279.236°C and 173.560°C, respectively, which implied that it possessed good thermal stability and was not easy to be degraded in processing. The higher T_c means higher crystallinity percentages of PHBV, it was supposed that there were some short segments of PHB in the polymer chains. Therefore, it was easy to synthesize PHBV block copolymers, and its strength would be enhanced as well. In summary, the PHBV produced by *R. rubrum* through the designed medium and method showed wide potential applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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