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ORIGINAL ARTICLE

# Mechanical properties of cold-drawn films of ultrahigh-molecular-weight poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) produced by *Haloferax mediterranei*

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## Abstract

We investigated the biosynthesis and properties of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) produced by *Haloferax mediterranei* NBRC14739$^T$ from glucose. When *H. mediterranei* grew on 5 g/L glucose in flask culture, 43.7 wt% PHBV with 12 mol% 3-hydroxyvalerate (3HV) accumulated intracellularly. Size-exclusion chromatography revealed that this polymer had a weight-average molecular weight ($M_w$) and polydispersity ($M_w/M_n$) of $4.7 \times 10^6$ g/mol and 1.7, respectively. Increasing the glucose concentration in the flask cultures slightly promoted cell growth and increased the PHBV content but had less effect on molecular weight. Scale-up cultivation in a 5-L jar yielded 4.0 g/L PHBV from 20 g/L glucose with a $M_w$ of up to $5.0 \times 10^6$ g/mol. These results showed that *H. mediterranei* can produce ultrahigh-molecular-weight (UHMW) PHBV from glucose. UHMW-PHBV (7 mol% 3HV, $M_w = 4.4 \times 10^6$ g/mol) was used to prepare cold-drawn films, the mechanical properties of which were characterized. The films were produced by tenfold cold drawing, followed by annealing at 100 °C, and had a tensile strength and Young's modulus of 258.7 MPa and 0.90 GPa, respectively.

## Introduction

Some environmental microbes accumulate polyhydroxyalkanoates (PHAs) for carbon and energy storage [1, 2]. PHAs are produced from renewable resources such as sugars, vegetable oils, and organic acids. Because PHAs from cells can serve as thermoplastic materials, they are used as carbon-neutral alternatives to petro-based thermoplastics. In addition, PHAs show superior biodegradability in natural environments and under physiological conditions, suggesting their potential for addressing the global problem of plastic waste [3, 4].

A representative PHA is poly(3-hydroxybutyrate) (PHB), which can be produced by numerous bacterial taxa. PHB is a highly crystalline polymer with a melting temperature ($T_m$) and glass transition temperature ($T_g$) of 180 °C and 4 °C, respectively [1]. Due to the low crystallization rate and a $T_g$ below the ambient temperature, PHB undergoes secondary crystallization, resulting in a hard but brittle material. To address this issue, the properties of PHB have been improved by copolymerization with other hydroxyalkanoates in vivo and by increasing the molecular weight to produce soft and tough materials.

The weight-average molecular weight ($M_w$) of PHAs produced by natural PHA producers is typically 0.1–2.0 × $10^6$ g/mol, depending on the strain and culture conditions. In contrast, ultrahigh-molecular-weight (UHMW) PHA has a defined $M_w$ of over $3.0 \times 10^6$ g/mol [5] and is formed by recombinant *Escherichia coli* strains harboring PHA biosynthetic genes from strain H16 of *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus* and *Ralstonia eutropha*) [6–8]. The PHB produced by these recombinant strains had a $M_w$ of up to $2.0 \times 10^7$ g/mol under optimized conditions. Iwata developed a two-step drawing method to produce strong fibers from UHMW-PHB, with a tensile strength of 1.3 GPa and a Young's modulus of over 10 GPa [9]. Aoyagi et al. developed a two-step drawing method to produce strong films from UHMW-PHB [10]. Although

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these processing technologies can overcome the brittleness of PHB, UHMW-PHAs have rarely been reported.

Recently, the biosynthesis of UHMW-PHB was further investigated both in recombinant *E. coli* and in natural PHB producers. Using recombinant *E. coli* strains harboring rearranged PHA synthesis genes from *C. necator*, Hiroe et al. demonstrated the formation of PHBs with a wide range of molecular weights [11]. The molecular weight of PHB produced in recombinant *E. coli* strains varies depending on the kind of PHA synthase gene expressed [12]. In addition, Castillo et al. reported PHB production by *Azotobacter vinelandii* ($M_w$ of $6.6 \times 10^6$ g/mol) [13]. In contrast, there have been few reports of UHMW-PHA copolymers. Arikawa et al. disrupted intracellular PHA depolymerase genes in recombinant *C. necator*, resulting in an increase in the $M_w$ of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) to up to $3.1 \times 10^6$ g/mol [14]. With mutants of PHA synthase from *Aeromonas caviae*, recombinant *C. necator* produced very high-molecular-weight PHB ($M_w = 3.68 \times 10^6$ g/mol) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanote) ($M_w = 3.59 \times 10^6$ g/mol) [15].

*Haloferax mediterranei*, a representative haloarchaeon capable of producing PHA, was isolated from a salt pond in Spain [16–18]. It grows and survives in media containing a high concentration of NaCl but is lysed in distilled water. It can produce PHA from a variety of carbohydrates—such as glucose, sucrose, and starch—as well as amino acids and organic acids [19]. The genetic constitution and metabolic pathways for PHA production in *H. mediterranei* have been reported [20–22]. Recently, it was reported that *H. mediterranei* produced PHAs from volatile fatty acids such as acetic acid and propionic acid; the $M_w$ of the PHAs was up to $3.6 \times 10^6$ g/mol [23]. This suggests the potential of *H. mediterranei* to produce UHMW-PHAs. In this study, we examined UHMW-PHA production from glucose instead of volatile fatty acids, which exert a toxic effect on the cells [16, 19]. A molecular weight and compositional analysis revealed the formation of UHMW-poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) by *H. mediterranei*. Finally, the copolymer was used to prepare cold-drawn films.

## Materials and methods

### Strains and culture media

*H. mediterranei* NBRC14739$^T$ and *C. necator* NCIMB11599, a glucose-assimilating mutant strain of the wild-type strain H16, were used as PHA producers. *H. mediterranei* was maintained at 37 °C on NBRC 257 agar (156 g/L NaCl, 13 g/L $MgCl_2 \cdot 6H_2O$, 20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $CaCl_2 \cdot 2H_2O$, 4 g/L KCl, 0.2 g/L $NaHCO_3$, 0.5 g/L KBr, 5 g/L yeast extract, 1 g/L glucose, and 20 g/L agar), and *C. necator* was maintained at 30 °C on nutrient-rich (NR) medium [24] plus 15 g/L agar. *H. mediterranei* was cultivated in modified basal synthetic (MBS) medium [19] (10 g/L glucose, 5 g/L yeast extract, 2 g/L $NH_4Cl$, 0.3 g/L $KH_2PO_4$, 0.005 g/L $FeCl_3 \cdot 6H_2O$, and marine salts [19] at ~25% final concentration) for seed culture and in MBS-P medium for PHA production. MBS-P medium is basically identical to MBS medium, except that it contains 0.0375 g/L $KH_2PO_4$. The pH of MBS and MBS-P was adjusted to 7.2 before sterilization by filtration through a 0.2 µm polyethersulfone membrane. A *C. necator* seed culture was prepared in NR medium, and PHA production was performed in nitrogen-limited mineral salt (MS-N) medium [24, 25].

### PHA production in flask cultures

Seed cultures of *H. mediterranei* were prepared in test tubes containing 3 mL of MBS medium for 2–3 days at 37 °C and 200 strokes/min on a BR-23FP bioshaker instrument (Taitec, Saitama, Japan). Cultivation was initiated by inoculation of 1 mL of seed culture into 50 mL of MBS-P medium containing a predetermined glucose concentration in a 300 mL Erlenmeyer flask. The flasks were shaken at 37 °C and 200 rpm for 72 h. After cultivation, 2 mL of culture broth was centrifuged in a plastic tube at 15,000 × *g* and 4 °C for 10 min. The pellet was washed with 20 wt% NaCl solution to remove the culture medium and then centrifuged again. The wash step was repeated twice, and the pellet was dried under reduced pressure. Another 1 mL of culture broth was centrifuged in a plastic tube, and the pellet was washed with distilled water to promote cell lysis. The cell suspension was again centrifuged, and the supernatant was discarded. The water-washing step was repeated twice, and the white pellet was dried under reduced pressure.

For PHB production by *C. necator*, 0.5 mL of a 24 h culture in NR medium was transferred to 50 mL of MS-N medium containing 5 g/L glucose. After 48 h of cultivation, the cells were harvested by centrifugation at 4000 × *g* and 4 °C for 10 min. The pellet was washed with deionized water, the suspension was centrifuged, and the cells were lyophilized to dryness.

### Analytical procedures

The content and composition of PHAs were determined by gas chromatography–mass spectrometry (GC–MS). Methanolysis of PHAs in dry cells was performed as described previously [25]. Methyl 3-hydroxyalkanoates were subjected to a 6890N Network GC system and 5973 Network Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA) equipped with a TC-WAX column (GL Sciences Inc., Tokyo, Japan). The temperature was set

at 80 °C for 2 min, increased to 230 °C in 10 min, and held at 230 °C for 2 min. Selected ionization monitoring mode at an *m/z* of 103 was used to quantify 3HB and 3-hydroxyvalerate (3HV) monomers. Authentic PHB and P (3HB-*co*-12 mol% 3HV) (Sigma-Aldrich, St. Louis, MO) were used to generate calibration curves.

The molecular weight of PHAs was determined by size-exclusion chromatography (SEC). Water-washed PHA pellets were dissolved in chloroform at 0.5 mg/mL and stirred overnight. After passing through a 0.45 µm polytetrafluoroethylene membrane, 10 µL of the sample solution was injected onto an HLC-8320GPC system (Tosoh Corporation, Tokyo, Japan) equipped with two TSKgel Super HZM-H columns (Tosoh). Chloroform was used as the eluent at 0.6 mL/min. The column was maintained at 40 °C during the analysis. Polystyrene with a $M_w$ of $5.89 \times 10^2$–$8.42 \times 10^6$ and narrow polydispersity (Tosoh) was used to generate a calibration curve.

The glucose concentration in the culture medium was determined by high-performance liquid chromatography (HPLC). First, 0.5 mL of culture broth was centrifuged to remove the cells and filtered through a 0.45 µm polyvinylidene difluoride membrane. Then, 20 µL of the sample was injected into a Waters 2695 HPLC system (Waters Corporation, Milford, MA) equipped with an SH-G guard column and Shodex SH1011 column (Showa Denko K. K., Tokyo, Japan) and a model 2414 refractive index detector (Waters). The mobile phase of 5 mM $H_2SO_4$ was used at 1.0 mL/min. During analysis, the columns were maintained at 60 °C. An authentic glucose standard was used to generate calibration curves.

The chemical structure of PHBV after purification by reprecipitation in excess methanol was analyzed by nuclear magnetic resonance (NMR). Samples were dissolved in $CDCl_3$ at 3 mg/mL, and $^1H$ and $^{13}C$ NMR spectra were recorded using a Bruker Advance III instrument (Bruker, Karlsruhe, Germany) at 400 MHz for $^1H$ NMR and 100 MHz for $^{13}C$ NMR.

Thermal analyses of PHBV were carried out by differential scanning calorimetry (DSC) using an SII DSC7020 (Hitachi High-Tech Science Corporation, Tokyo, Japan) at a heating rate of 10 °C/min. The thermal program was from −70 °C to 200 °C for the first scan, followed by a hold for 2 min, quenching at −70 °C, and heating to 200 °C for a second scan.

### Jar fermenter experiment

A seed culture of *H. mediterranei* was prepared in a test tube containing 3 mL of MBS at 37 °C and 200 rpm for 2–3 days, and two 1 mL aliquots of the culture broth were transferred to two 300 mL flasks containing 50 mL of MBS. After 24 h of cultivation at 37 °C and 200 rpm, 100 mL of the culture was transferred to 2.0 L of MBS-P containing 20 g/L glucose in a 5 L jar fermenter (Marubishi Bioengineering Co., Ltd, Tokyo, Japan). The agitation speed was controlled to maintain a dissolved oxygen level of 100% during cultivation. The temperature was maintained at 37 °C. The pH of the culture was set at 7.2, and 12% $NH_3$ aq was added when the pH decreased below 7.0. After cultivation, the cells were harvested by centrifugation, and the pellet was washed with water to collect the PHAs. After drying, the pellets were further purified by precipitation from chloroform solution into excess methanol and drying under reduced pressure for 3 days at room temperature.

### Uniaxial cold drawing and annealing

Solvent-cast films of PHBV were prepared from chloroform solution and dried under reduced pressure for 3 days. The dried films were cut into 10 × 30 mm pieces and used for cold-drawing experiments, as reported previously [26]. The test specimen was melted at 170 °C or 200 °C for 30 s and quenched in ice-cold water. The amorphous films were stretched tenfold in ice-cold water using a hand-drawing machine and annealed in an oven at various temperatures for 2 h to promote crystallization. Before tensile testing, the films were incubated at room temperature for at least 3 days.

### Tensile strength testing

The tensile strength was measured using an ES Test ES-LX (Shimadzu Corporation) with a strain rate of 20 mm/min. Three measurements were performed for each condition.

## Results and discussion

### Production of UHMW-PHBV by *H. mediterranei*

We first cultivated *H. mediterranei* on 5 g/L glucose to produce PHBV (Table 1) and analyzed its molecular weight. Figure 1 shows the SEC elution profiles of PHBV produced by *H. mediterranei* and of PHB produced by *C. necator* NCIMB11599, with commercially available PHB used as a reference. The PHBV peak for *H. mediterranei* began at 6.5 min and reached its maximum at 7.1 min, whereas the PHB peak for *C. necator* began at approximately 6.8 min and reached its maximum at 7.6 min. The $M_n$ and $M_w$ were calculated to be 2.7 and $4.7 \times 10^6$ g/mol, respectively, for PHBV from *H. mediterranei* and 0.7 and $1.6 \times 10^6$ g/mol, respectively, for PHB from *C. necator*. The SEC-based relative $M_w$ of PHB was correlated with the absolute $M_w$ of PHB (absolute $M_w = 0.7 \times$ SEC-based relative $M_w$) [5]. Therefore, *H. mediterranei* likely produced UHMW-PHBV from glucose.

**Table 1** Biosynthesis of PHBV from glucose by *H. mediterranei*[a]

| Glucose | Dry cell weight | PHBV content[b] | Composition[b] | | Molecular weight[c] | | |
|---|---|---|---|---|---|---|---|
| | | | 3HB | 3HV | $M_n$ | $M_w$ | $M_w/M_n$ |
| (g/L) | (g/L) | (wt%) | (mol%) | (mol%) | ($\times 10^6$) | ($\times 10^6$) | |
| 5 | 4.2 ± 0.6 | 43.7 ± 4.2 | 88 ± 0.6 | 12 ± 0.6 | 2.8 ± 0.1 | 4.7 ± 0.2 | 1.7 |
| 10 | 5.5 ± 0.3 | 45.8 ± 2.1 | 88 ± 0.3 | 12 ± 0.3 | 2.6 ± 0.5 | 4.0 ± 0.7 | 1.6 |
| 20 | 5.1 ± 0.5 | 46.5 ± 2.0 | 87 ± 0.7 | 13 ± 0.7 | 3.0 ± 0.2 | 4.5 ± 0.2 | 1.5 |
| 50 | 5.0 ± 0.4 | 46.4 ± 2.0 | 87 ± 0.1 | 13 ± 0.1 | 2.7 ± 0.3 | 4.1 ± 0.4 | 1.5 |

[a]Cells were cultivated in MBS media with the indicated glucose concentrations at 37 °C for 72 h. Data are means ± standard deviations from at least three independent experiments

[b]Determined by GC–MS

[c]Determined by SEC

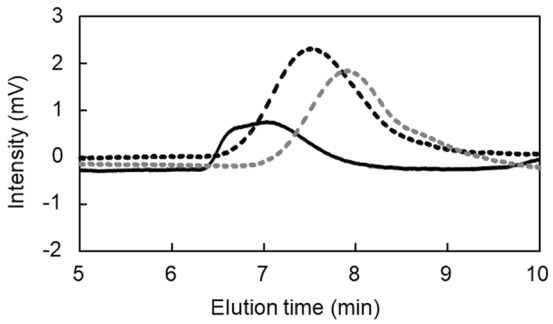


**Fig. 1** Elution profiles of PHAs by size-exclusion chromatography. Black solid line, PHBV synthesized by *H. mediterranei* NBRC14739; black dashed line, PHB synthesized by *C. necator* NCIMB11599; gray dashed line, PHB purchased from Sigma

The molecular weights of PHBV produced by *H. mediterranei* were analyzed. Han et al. reported that PHBV produced from 10 g/L glucose by *H. mediterranei* had $M_w$ and $M_w/M_n$ values of $1.56 \times 10^6$ g/mol and 1.41, respectively [27]. Koller et al. demonstrated that *H. mediterranei* grown on whey produced PHBV with a $M_w$ and $M_w/M_n$ of $696 \times 10^3$ g/mol and 2.2, respectively [28]. Don et al. reported that *H. mediterranei* produced mixtures of PHBV with two different molecular weights of $569.5 \times 10^3$ and $78.2 \times 10^3$ g/mol [29]. We produced PHBV with a $M_w$ of $4.7 \times 10^6$ g/mol, indicating that the molecular weight of PHBV produced by *H. mediterranei* varies according to the culture conditions. In general, the molecular weight of PHA is affected by the concentration of PHA synthase, the chain-transfer reaction, the catalytic activity of PHA synthase, and the degradation of PHA during biosynthesis [30]. To control the molecular weight of PHA, it is necessary to identify the primary determinant thereof by investigating the expression levels of PHA synthase genes, the intracellular concentration of chain-transfer reagents, and the metabolic pathways and fluxes. Notably, *H. mediterranei* contains a PHA synthase comprising two subunits: $PhaE_{Hm}$ and $PhaC_{Hm}$. Three homologous genes of PhaC are present in the genome of *H. mediterranei*, and alteration of the PhaC subunit modulates the molecular weight of PHBV [31]. Therefore, it is necessary to assay the polymerization activity of PHA synthases with various PhaC subunits. Additionally, we observed a bimodal profile of PHBV produced by *H. mediterranei* (Fig. 1, black solid line). The MBS medium used in this study contains yeast extract in addition to glucose. The use of a complexed medium might result in the formation of two kinds of polymers with different molecular weights, as also observed in a previous report [29].

## Effect of glucose concentration on PHBV production in flask cultures

HPLC analysis of the culture medium of *H. mediterranei* after 72 h revealed exhaustion of the 5 g/L glucose. We note that the obtained biomass (4.2 g/L DCW) in the 5 g/L glucose medium is too high considering the assimilation of glucose as a sole carbon source. This implies that not only glucose but also yeast extract could be assimilated for the growth and synthesis of PHBV by *H. mediterranei* because MBS medium contains 5 g/L yeast extract. In fact, *H. mediterranei* grew and accumulated PHBV in NR AS-168 medium that contained 5 g/L yeast extract and 5 g/L casamino acids [20]. As shown in Table 1, the dry cell weight (DCW) reached 5.5 g/L with 10 g/L glucose and decreased slightly with increasing initial glucose concentration. The PHBV content and monomer composition were similar irrespective of the initial glucose concentration. The highest PHBV concentration, 2.5 g/L, was achieved when the cells were cultivated on 10 g/L glucose. The $M_w$ values of PHBV were $\geq 4.0 \times 10^6$ g/mol, and the highest $M_w$ of $4.7 \times 10^6$ g/mol occurred in the presence of 5 g/L glucose. HPLC analysis after 72 h of cultivation revealed a maximum glucose consumption of 7.5 g/L in cultures with initial glucose concentrations of 10 and 20 g/L. We observed no further consumption of glucose at 144 h with an initial glucose concentration of 20 g/L (data not shown). These results suggested that glucose consumption limited cell growth and PHBV production in flask cultures.

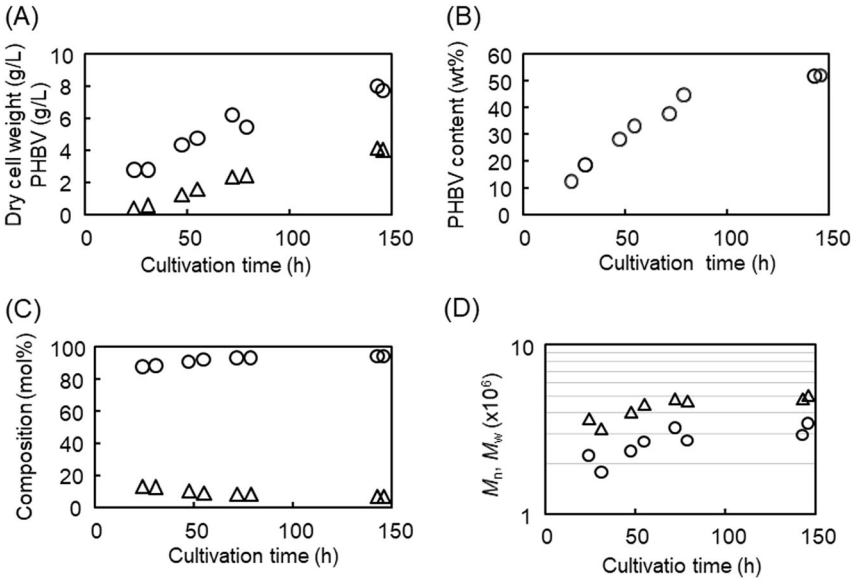


**Fig. 2** Time-dependent changes in PHBV production by H. *mediterranei* in a jar fermenter. Dry cell weight (**a**, open circles), PHBV (**a**, open triangles), PHBV content (**b**), compositions of 3HB (**c**, open circles) and 3HV (**c**, open triangles) in PHBV, and $M_n$ (**d**, open circles) and $M_w$ (**d**, open triangles)

In a previous report, volatile fatty acids were used as promising substrates for high-molecular-weight PHBV production in *H. mediterranei* [23]. However, it took a long time (96–335 h) for cultivation, yielding PHBV with a low concentration (0–1.5 g/L PHBV). By contrast, our cultivation in which glucose was used as a carbon source yielded 1.8–2.5 g/L PHBV in 72 h. In addition, UHMW-PHBV formation was achieved in the absence of fatty acids. These results imply that UHMW-PHBV is produced regardless of carbon sources in *H. mediterranei* and that glucose seems to be suitable for UHMW-PHBV production by *H. mediterranei*.

## PHBV production in jar fermenters

To promote PHBV production, jar fermenter cultivation with regulation of pH was performed using 2.0 L of MBS-P containing 20 g/L glucose. As shown in panels a, b of Fig. 2, DCW and PHBV concentrations increased with cultivation time, reaching 7.7 and 4.0 g/L after 146 h of cultivation, respectively, with a PHA content of 51.7 wt%. At the end of cultivation, glucose in the culture broth was completely exhausted (data not shown). The 3HV content in the polymer decreased gradually from 13 to 6 mol% (Fig. 2c), whereas the molecular weight increased with time. The $M_w$ of PHBV was 3.6 and $5.0 \times 10^6$ g/mol at 24 and 146 h, respectively (Fig. 2d). The yield of PHBV from glucose was 0.20 g-PHBV/g-glucose. Therefore, UHMW-PHBV can be produced from glucose using a jar fermenter with pH regulation, as well as in flasks.

The monomer-supplying pathways for 3HV in *H. mediterranei* were proposed to be linked to amino acid metabolism [22]. One possibility to account for the time-dependent changes in the 3HV content in the polymer observed in our study is changes in the amount of yeast extract, which contains amino acids/peptides. *H. mediterranei* was reported to grow well on yeast extract and casamino acids [20]. In addition, HPLC measurements revealed 17.3, 12.9, and 8.4 g/L glucose left in the culture broth at 24, 48, and 72 h, respectively. Therefore, in the early stage of jar cultivation, sufficient yeast extract in MBS medium would promote 3HV supply into PHBV (13 mol% 3HV at 24 h, Fig. 2c). With cultivation time, a decrease in yeast extract (but glucose was still available) narrowed the 3HV supply, resulting in a relatively low 3HV content (7 mol% 3HV at 72 h, Fig. 2c) in the polymer. This could also account for the differences in the 3HV content in PHBV between flask cultivation and jar fermentation.

Similar to the elution profile of PHBV from flask culture (black solid line in Fig. 1), bimodal elution of PHBV from jar fermentation was observed. As shown in Fig. 3, a high-molecular-weight fraction (elution time between 6.5 and 7.0 min) in the elution profile increased with cultivation time. In batch cultivation, the contents of nutrients decrease with cultivation time, and then some are exhausted in the latter period of culture. However, as mentioned above, glucose was continuously consumed through cultivation in our jar experiment. Therefore, it could be speculated that glucose contributes to the formation of the high-molecular-weight fraction.

After purification by precipitation in methanol, 6.7 g of PHBV was recovered from 2 L of culture as a white solid. PHBV was analyzed by NMR and SEC. The $^1$H NMR spectrum confirmed the 3HV structure and that 3HB was the dominant component (data not shown). The molar fraction of 3HV was calculated to be 7.0 mol%. The $M_w$ and $M_w/M_n$ of PHBV after purification were $4.4 \times 10^6$ g/mol and 1.6, respectively. DSC revealed that the $T_m$ and $T_g$ values of PHBV were 152.0 °C and 1.7 °C, respectively. Compared

with the $T_m$ of PHB at ~180 °C, the $T_m$ of PHBV was more than 20 °C lower. This likely inhibited the decrease in molecular weight during melting, thus improving the mechanical properties of the films (see below).

## Physical properties of cold-drawn films of UHMW-PHBV

To assess the potential of UHMW-PHBV for films with a high tensile strength, cold-drawn films were prepared as described previously [26]. A tenfold drawing ratio was applied with no breaking of the films. The thicknesses of the drawn films were 83.0–114.9 μm (Table 2). Figure 3 shows the relationship between tensile strength and the annealing temperature of cold-drawn PHBV films melted at 170 °C and 200 °C. The tensile strength increased with annealing temperature, peaking at 258.7 MPa when melted and annealed at 170 °C and 100 °C, respectively. This maximum strength was 8.7-fold higher than that of the UHMW-PHBV cast film with no drawing (29.9 MPa, run 0 in Table 2). This sample (run 4 in Table 2) showed a Young's modulus of 0.90 ± 0.02 GPa and 49.4 ± 4.7% elongation to break. The tensile strength of cold-drawn UHMW-PHBV films was comparable to that of cold-drawn UHMW-PHB (237 MPa) [26]. In contrast, the PHBV films melted at 200 °C had a lower tensile strength than the PHBV films melted at 170 °C (Fig. 4). The film melted and annealed at 200 °C and 100 °C, respectively, had the highest Young's modulus but poor tensile strength and elongation (run 6 in Table 2).

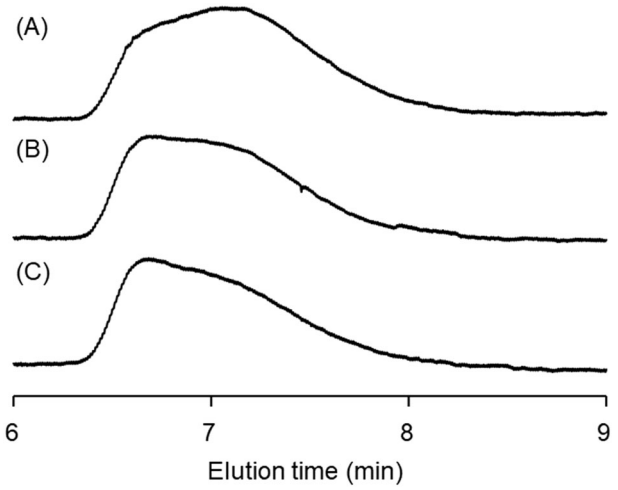


**Fig. 3** Comparison of the elution profiles of PHBV from the jar fermenter experiment by size-exclusion chromatography. Cells were taken from a jar fermenter at (**a**) 48 h, (**b**) 72 h, and (**c**) 146 h and processed for molecular weight analysis. The $M_w$s of the samples were (**a**) $4.0 \times 10^6$ g/mol, (**b**) $4.8 \times 10^6$ g/mol, and (**c**) $5.0 \times 10^6$ g/mol

We then measured the DSC of cold-drawn and annealed films (runs 2–5 in Table 2) to further characterize the crystallinity of the films. The theoretical enthalpy of fusion of a 100% crystalline PHB (146 J/g) was used to estimate the crystallinity of UHMW-PHBV containing 7 mol% 3HV [32]. As shown in Table 2, the solvent-cast film (run 0) had a crystallinity of 54.9%, whereas the cold-drawn and annealed films (runs 2–5) had crystallinities of 60.6–63.7%. We could not observe distinct differences in crystallinity dependent on annealing temperatures. These data suggested that increasing the crystallinity by the cold-drawing and annealing process resulted in higher tensile strength of UHMW-PHBV films, similar to that of UHMW-PHB [26]. It will be necessary to investigate crystalline structures and

**Table 2** Processing parameters and physical properties of UHMW-PHBV[a] cold-drawn and annealed films[b]

| Run | Melting (°C) | Annealing (°C) | Thickness (μm) | Tensile strength (MPa) | Young's modulus (GPa) | Elongation at break (%) | Crystallinity[c] (%) |
|---|---|---|---|---|---|---|---|
| 0 | NA[d] | NA | 128.8 ± 12.0 | 29.9 ± 0.3 | 1.7 ± 0.1 | 3.5 ± 0.2 | 54.9 |
| 1 | 170 | 25 | 102.9 ± 1.9 | 210.9 ± 21.0 | 1.18 ± 0.05 | 49.5 ± 2.6 | –[e] |
| 2 | 170 | 60 | 88.6 ± 0.6 | 253.7 ± 40.2 | 1.26 ± 0.07 | 30.0 ± 3.5 | 60.9 |
| 3 | 170 | 80 | 87.7 ± 0.2 | 245.9 ± 29.6 | 1.23 ± 0.10 | 37.6 ± 5.3 | 63.7 |
| 4 | 170 | 100 | 83.0 ± 0.2 | 258.7 ± 26.0 | 0.90 ± 0.02 | 49.4 ± 4.7 | 60.8 |
| 5 | 170 | 120 | 93.0 ± 0.2 | 201.2 ± 18.6 | 1.08 ± 0.06 | 54.8 ± 3.2 | 60.6 |
| 6 | 200 | 100 | 114.9 ± 3.3 | 122.3 ± 12.9 | 1.84 ± 0.28 | 38.1 ± 5.0 | – |
| 7 | 200 | 120 | 99.0 ± 1.0 | 145.5 ± 3.8 | 1.12 ± 0.06 | 55.5 ± 0.5 | – |

[a]UHMW-PHBV was composed of 93 mol% 3HB and 7 mol% 3HV, as determined by $^1$H NMR. The $M_w$ and $M_w/M_n$ values of UHMW-PHBV were $4.4 \times 10^6$ g/mol and 1.6, respectively, as determined by SEC

[b]Data are means ± standard deviations from three independent measurements

[c]Crystallinity ($X_c$) was estimated by the following equation: $X_c\ (\%) = \Delta H_m/\Delta H_m^* \times 100$, where $\Delta H_m$ is fusion enthalpy of the samples, as determined by DSC, and $\Delta H_m^*$ is the theoretical fusion enthalpy of a 100% crystalline PHB (146 J/g).

[d]Not applicable

[e]Not determined

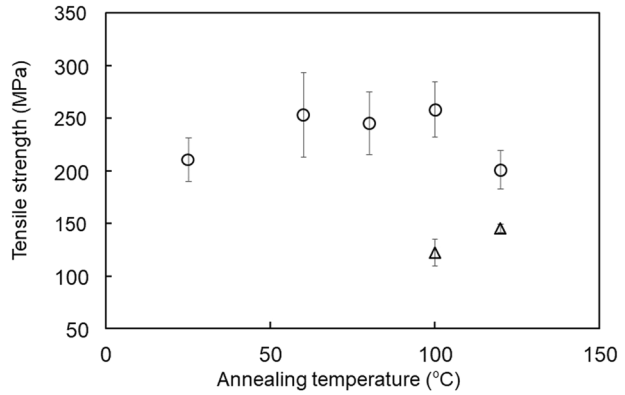


**Fig. 4** Tensile strength of cold-drawn films as a function of annealing temperature. The films were melted at 170 °C (open circles) and 200 °C (open triangles) during preparation. The bars represent standard deviations from three independent measurements

orientations in detail using X-ray diffraction patterns. There were no clear differences in transparency between cast film and other cold-drawn films (data not shown).

Iwata and Doi reported that tenfold cold-drawn films of PHBV (8 mol% 3HV) with an absolute $M_w$ of $1.0 \times 10^6$ g/mol showed a tensile strength of 117 MPa when melted and annealed at 180 °C and 75 °C, respectively [33]. This implies that the molecular weight of PHBV influences the mechanical properties of cold-drawn PHBV films. In addition, differences in the temperatures of the melting process lead to differences in molecular weight. Random chain scission during thermal degradation of PHB begins below its $T_m$ (~180 °C), and its rate increases with temperature [34]. Indeed, the $M_w$ of processed PHBV films in this study was $0.91 \times 10^6$ g/mol for samples melted at 200 °C and annealed at 100 °C and $1.8 \times 10^6$ g/mol for samples melted and annealed at 170 °C and 100 °C, respectively. Therefore, PHBV films melted at 200 °C undergo chain scission more rapidly than those melted at 170 °C, leading to differences in molecular weight. Our results suggest that UHMW-PHBV has potential for practical, strong films.

## Conclusion

For the first time, we demonstrated the biosynthesis of UHMW-PHBV from glucose by *H. mediterranei*. The PHBV produced in flask cultures comprised 12–13 mol% 3HV and had a $M_w$ of 4.0–4.7 × $10^6$ g/mol, depending on the cultivation conditions. The UHMW-PHBV (7 mol% 3HV, $M_w = 4.4 \times 10^6$ g/mol) obtained by jar fermenter cultivation was used to produce cold-drawn and annealed films with a tensile strength of 258.7 MPa, comparable to that of a film produced from UHMW-PHB. Therefore, *H. mediterranei* shows promise for commercial production of UHMW-PHBV for use not only in commodity products but also in specific applications that require high strength.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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