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Polyhydroxybutyrate and Hydroxyvalerate Production by *Bacillus megaterium* Strain A1 Isolated from Hydrocarbon-Contaminated Soil

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ABSTRACT: In this study, we attempted to find an alternative microbial resource as a bioplastic producer. Among all of the isolates, the A1 strain produced 44% poly(β-hydroxybutyrate) (PHB) in proportion to its dry cell weight. The molecular identification of the 16S RNA gene showed that this bacterium was a strain of *Bacillus megaterium* with the accession number KC579390. The optimization studies led us to the conclusion that the highest poly(β-hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) production was 78% when 5% molasses was used as the carbon source at pH 6 and 35°C after 60 h of incubation. Attenuated total reflectance Fourier transform infrared (FTIR) spectroscopy and H-NMR were used for chemical characterization. Differential scanning calorimetry was used to determine the thermal properties of the PHB and PHBV that were synthesized with sucrose and molasses as carbon sources, respectively. The FTIR spectra of the polymers were characterized by typical absorption bands at 1715–1720 cm^{-1} for amide-bound C═O bands and 1261–1279 cm^{-1} for an ester-bound C—O band. The molecular weights of PHB and PHBV synthesized with sucrose and molasses were calculated as 428 and 498 kDa, respectively, according to the viscometric method. This study indicated that the *B. megaterium* strain A1 is an alternative microbial resource as a bioplastic producer. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40530.

KEYWORDS: biocompatibility; biodegradable; biomaterials; biopolymers & renewable polymers; biosynthesis of polymers

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INTRODUCTION

Nowadays, the production of biodegradable plastics has gained great importance in the prevention of environmental damage caused by petroleum-based plastics.[1] Poly(β-hydroxybutyrates) (PHBs) are biodegradable and biocompatible materials. PHB is the most common form of poly(β-hydroxyalkanoate) in nature. PHBs are formed by various microorganisms under production conditions with nutrient limitations.[2,3] Bioplastic accumulation generally occurs in the presence of excess carbon sources but also in the absence of the nutrients required for growth, such as nitrogen, oxygen, phosphorus, sulfur, magnesium, potassium, and iron.[2–7]

The determination of the physical and chemical properties of PHBs is a crucial factor in determining the application areas of these polymers. PHBs are generally spherical, and each granule is between 100 and 800 nm. Each is surrounded by a nonunitary 2–4-nm thick membrane. Approximately 98% of the granules contain PHB, and 2% contain protein.[2,8] The melting temperature (T_m) of PHB, a solid but not fragile material, is 157–188°C, which is close to the temperature at which the synthetic polymer thermally decomposes. PHB is a polymer that can be shaped by pressing, as it is a thermoplastic.[2,9,10] It is known that the molecular weight of the polymer is between 60.000 and 2.000.000 Da, depending on the type of bacteria, growth conditions, and cell life cycle.[11] However, the hardness of PHB, a thermoplastic, is four times higher than that of polyethylene. Also, the dilation feature of PHB for breaking is 6%, whereas that of polypropylene is 400%. The usage areas of PHBs are restricted because of these physical properties. To eliminate this disadvantage and to increase the potential for use, PHB copolymers Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) that contain 3-hydroxyvalerate (3HV) units have been developed. Thus, polymers have become more robust and flexible. There have been studies reported in the literature that show that as 3HV units increase, the flexibility also increases.[12–14]

The areas of use for PHBs produced by bacteria are determined by their physical and chemical properties. One can produce PHBs with new characteristics by chancing the carbon source at

the stage of biosynthesis to increase the areas of use for PHBs. Polymers with different structures and properties can be synthesized with different carbon sources used by PHB producer microorganisms.[1,15]

The high costs of the synthetic carbon sources used in the production of PHB are a limiting factor in the commercial success of PHB production. Studies on recombinant species have been performed to reduce PHB production costs. Also, there have been studies on strains that obtain a high PHB yield with industrial waste carbon sources.[16]

In this study, bacteria isolation was carried out from hydrocarbon-contaminated soils in the Eskisehir Organized Industrial Zone, and the PHB production capabilities of the isolated microorganisms were investigated. Molecular techniques have been used for species identification of the most effective isolates in PHB production. In addition, optimization studies were carried out to increase PHB production and to determine the best production conditions. Characterization studies were performed to determine the physical and chemical properties of the produced PHB.

EXPERIMENTAL

Isolation Studies

Areas determined in the Eskisehir Organized Industrial Zone and used for a long time as receiving environments for derivatives of hydrocarbon were selected for isolation studies. The soil sample (10 g), which was obtained from different hydrocarbon-contaminated stations under aseptic conditions, was taken and suspended in 90 mL of a sterile normal saline solution (0.9%), and serial dilutions were made up to 10^{-5} from these solutions. We obtained minimal salt agar cultures with the spread-plate method by taking 100 μL from each dilution. After a 48-h incubation period at 35°C, single formed colonies with different morphological appearances were selected and cultured in separate Petri dishes, and pure cultures of the isolates were obtained. The isolates were stored at +4°C.

Determination of the PHB Production Capabilities of the Isolates

Stock cultures were cultured in 50-mL volume of nutrient broth (NB), and the cultures were activated by incubation for 48 h at a 150-rpm agitation speed at 35°C. A vaccine culture was prepared from the activated cultures, and culturing was performed in a 100-mL volume of NB medium with a 2% v/v ratio in a 250-mL Erlenmeyer flask. The culture was incubated at 35°C for 48 h at 150 rpm.

After the isolates were cultivated in the NB medium, the polymer medium, which contained a high amount of carbon source for the isolates to produce PHB and the limited essential nutrients needed for growth, was prepared. The polymer medium contained the following: 20 g/L sucrose, 1.5 g of K_2HPO_4, 4.7 g/L $Na_2HPO_4{\cdot}2H_2O$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $CaCl_2{\cdot}2H_2O$, 0.1 g/L citric acid, and 1 mL of trace element solution. The trace element solution consisted of 0.2 g of $FeSO_4{\cdot}7H_2O$, 0.3 g/L H_3BO, 0.2 g/L $CoCl_2{\cdot}6H_2O$, 0.03 g/L $ZnSO_4{\cdot}7H_2O$, 0.03 g/L $MnCl_2{\cdot}4H_2O$, 0.03 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.03 g/L $NiSO_4{\cdot}7H_2O$, and 0.01 g/L $CuSO_4{\cdot}5H_2O$. To transfer the isolates grown in NB to the polymer medium vaccine, the culture was centrifuged at 6000 rpm for 20 min. The pellets obtained from the centrifuge process was transferred to the polymer medium. The isolates transferred to the polymer medium were incubated for 48 h in a shaking incubator that had been set up at 35°C and 150 rpm.[17]

PHB Extraction and Measurement

After incubation, the polymer medium was centrifuged in sterile centrifuge tubes at 6000 rpm for 20 min. The pellets were obtained after the centrifuge was kept at −80°C overnight. To determine the dried biomass, the pellets were dried at 50°C. After the pellets dissolved, they were vortexed through mixing with 5 mL of sodium hypochlorite. Then, they were kept in a shaking water bath overnight at 37°C. The supernatant part was removed by the centrifugation of the pellet–sodium hypochlorite mix at 6000 rpm for 20 min. A volume of 5 mL of ethanol was added to the pellets, and they were mixed with a vortex to extract lipids and other molecules. Then, the supernatant was removed by centrifugation at 6000 rpm for 20 min. A volume of 5 mL of chloroform was added to the remaining pellets, and after the tubes were mixed with a vortex, they were kept in a shaking water bath at 60°C until the PHB dissolved. After this step, 1 mL was taken from each tube in which PHB extraction had taken place and kept in incubator at 60°C until the chloroform was completely removed. The extracted PHB with chloroform removed changed from a liquid phase to a crystalline phase. After 5 mL of H_2SO_4 was added, the crystallized PHB was kept in a water bath at 100°C for 10 min. After cooling, the absorbance values of the samples taken from the water bath were read in a spectrophotometer (UV-2550, Shimadzu) at a 235-nm wavelength. All of the measurements are given as percentage of PHB and formulated as follows:[18]

$$\text{PHB (\%)} = (\text{PHB concentration} \times 0.1)/(\text{Dried biomass} \times 10) \quad (1)$$

Molecular Identification of the Isolates

The 16S rRNA product was provided by the polymerase chain reaction (PCR) and was amplified from genomic DNA with universal primers for bacteria. For the extraction of genomic DNA belonging to the PHB-producing isolate, 200 μL of liquid culture was boiled for 15 min at 95°C and centrifuged for 5 min at 13,000 rpm, and the obtained supernatant was used as a template for the PCR reactions. The reaction mixtures contained 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-Hydrochloride (pH 9), 200 μM of each deoxyribonucleotide triphosphate (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, Invitrogen Corp., Carlsbad, CA), 1 U of Taq DNA polymerase (Invitrogen), 0.2 m*M* of each oligonucleotide primer, and 50 ng of template DNA in a total volume of 50 μL. The sequence of the forward primer was 27f 5′-AGAGTTTGATCATGGCTCAG-3′, and that of the reverse primer was 1492r 5′-GGTTACCTTGTTACGACTT-3′.[19] The following conditions were used for amplification: a cycle of 94°C for 3 min, 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min, and an extension step of 7 min at 72°C. The PCR product was purified with a Wizard Genomic DNA purification kit (Promega). The purified PCR product was sequenced with the GenomeLabDTCS Quick Start Kit and a Beckman Coulter CEQ8000

Genetic Analyzer (Beckman Coulter). The used primer sets were 341f 5′-CCTACGGGAGGCAGC AG-3′ and 907r 5′-CCGTCAATTCTTTTGAGTTT-3′.[20] The 16S rRNA gene sequences were initially compared with reference sequences at National Center for Biotechnology Information (NCBI)[21] with Basic Local Alignment Search Tool (BLAST).[22]

Optimization Studies

To increase the efficiency of the production of PHB, the effect of different carbon sources on PHB production was researched, and parameters such as the amount of carbon source, pH of the medium, incubation temperature, and incubation time were optimized.

For the purpose of determining the effects of the different carbon sources on the PHB production, the effects of the synthetic and industrial waste carbon sources were investigated. For this purpose, the polymer medium was modified to determine the effect of the synthetic carbon sources on PHB production. The PHB that was obtained with the addition of 20 g/L glucose, fructose, mannitol, glycerol, starch, and maltose into the polymer medium and the PHB that was produced in the polymer medium with sucrose were compared in terms of efficiency.

In the study carried out to determine the effect of industrial wastes on PHB production, molasses and vinasse from Eskisehir Sugar Factory, olive black water with three phases from an olive oil factory in the district of Edremit in Balıkesir, and whey from a dairy near Balıkesir were provided. By the addition of 3% of these industrial waste carbon sources, the PHB production yields were evaluated. We continued the study by changing the selected carbon source amount within the range 1–30%. In addition, the effects of the pH of the medium (pH 4, 5, 6, 7, 8, and 9), incubation temperature (25, 30, 35, and 40°C), and incubation time (12, 24, 36, 48, 60, and 72 h) on PHB production were investigated. Each experiment was repeated at least three times and independently for all parameters. The PHB production yields were evaluated in terms of the amount of PHB produced for each dry biomass.

Characterization

A PerkinElmer Spectrum One Nicolet 520 Fourier transform infrared (FTIR) spectrometer was used for characterization, and the tests were performed at ambient temperature. The FTIR spectra of the prepared samples were determined within the range 400–4000 cm^{-1}. In this study, the FTIR analyses of the PHB produced with *Alcaligenes eutrophus* DSM 545 and commercial PHB were also carried out similarly.

^{1}H-NMR (Bruker 400-MHz AV) studies in CH_3COCH_3-d_6 at 27°C, were performed to determine the molecular structures of PHB and PHBV. The compositions of the synthesized copolymers with molasses were determined by NMR data by a comparison of the integrals of the methyl group regions in the spectra of 3-hyroxybutyrate (3HB) and 3HV units according to the following equation:[23]

$$m_1 = m_2 = n_2 A_{m1}(CH3HB) = n1Am2(CH3HV) \quad (2)$$

where m_1 is number of —CH3 groups in single 3-hydroxybutryrate monomer, which is "1", m_2 is number of —CH3 groups in single 3-hydroxyvalerate monomer, which is "1", "CH3 HB" —CH3 groups in single 3-hydroxybutryrate monomer, "CH3 HV" —CH3 groups in single 3-hydroxyvalerate monomer, A_{m1} and A_{m2} are the normalized areas per hydrogen from the corresponding functional groups of the monomer unit regions in the ^{1}H-NMR spectra and n_1 and n_2 are the integers of the proton(s) in the functional groups of the monomers. The monomer unit ratio ($m_1 + m_2$) was 1.

The viscosimetric molecular weights of PHB and PHBV were determined. The polymer solutions (in chloroform) and chloroform were loaded into a Ubbelohde capillary viscometer, and the flow times were measured at 25°C. The Viscosity average molecular weight (M_v) values of PHB and PHBV were calculated with the Mark–Houwink–Sakurada equation:

$$[n] = K \times M_v^{\alpha}$$

where K is a constant that for PHB is 0.019 and for PHBV is 0.0118 and α is a constant value that is 0.78 for each polymer.[24] $[n]$ is intrinsic viscosity (of polymer solution (in chloroform)).

The T_m and melting enthalpy (ΔH_m) values of the polymers were determined by differential scanning calorimetry (DSC; DSC-60 Shimadzu). Samples, as cast films, of 2–3 mg were weighed into hermetic aluminum pans and analyzed.[25]

RESULTS

Isolation Studies and PHB Production Capabilities of the Isolates

Even though hydrocarbon-contaminated areas are rich in carbon and its derivatives, they have limited compositions of nitrogen and other trace elements. Therefore, they are selected as habitats in which potential PHB producers could exist. The soil samples were taken from the Eskisehir Organized Industrial Zone for bacteria isolation from hydrocarbon-contaminated soils. Forty-two different isolated samples were purified in isolation studies.

The PHB production capabilities of the 42 isolates that were obtained as a result of the isolation study were scanned. The results of this study demonstrated that 36 of these 42 isolates were capable of producing trace amounts of PHB, and 6 of them were capable of producing more than 1% PHB. In this study, the PHB yields were obtained as follows: 44% for A1, 4% for B1, 1% for B2, 8% for B5, 3% for B10, and 3% for B27.

Identification of the Most Active Isolate in Terms of PHB Production

The 42 isolates were evaluated for the production of PHB. As a result of this study, after we determined that the A1 isolate was the most effective isolate in proportion to the dry cell weight in terms of PHB production ability, molecular techniques were used for the species identification of the isolates.

The partial 16S rRNA gene sequence of the PHB producer strain was determined. These nucleotide sequences were deposited in the Gen-Bank database under accession number (KC579360). In this study, the strain was named *Bacillus megaterium* strain A1.

Determination of the Optimum Conditions

To investigate the effects of different carbon sources on the PHB production, synthetics, such as glucose, fructose, mannitol,

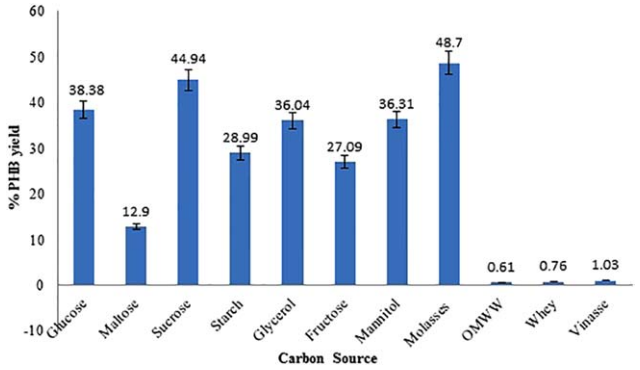

Figure 1. PHB yields (%) in proportion to the dry cell weight observed in the study carried out with synthetic and industrial waste carbon sources (working conditions: pH 7.0, 35°C, 150 rpm, and 48 h). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

glycerol, starch, maltose, and industrial wastes, including molasses (3% v/v), olive mill wastewater (3% v/v), whey (3% v/v), and vinasse (3% v/v), were tested as carbon sources. The study results are given in Figure 1. As a result of the study, the PHB yield in proportion to the dry cell weight was observed as 48% in the medium, which contained a maximum of 3% molasses on average.

With the purpose of determining the effect of the carbon source on PHB production, we pursued the PHB production quantities in media with 1–30% molasses. As shown in Figure 2, the highest PHB production of 52% was achieved in the medium with 5% molasses.

To determine the effect of the pH value on PHB production, a medium with 5% molasses, in which the pH was adjusted from 4 to 9, was prepared. Figure 2 shows the results of this study. The highest PHB yield in the study was determined to examine the effect of pH was determined as 75% at pH 6 in proportion to dry cell weight.

To determine the effect of temperature on PHB production, an investigation was carried out in the medium with 5% molasses and at pH 6 at 25–40°C medium temperatures for 48 h (Figure 2). In this study, the best PHB production yield of 72% in proportion to the dry cell weight was determined at 35°C. Under the same conditions, for the purpose of determining the effect of the incubation time on PHB production, the effect of the incubation time was investigated for 12–72 h. The results of the PHB production up to 72 h are presented in Figure 2. In this study, a maximum PHB production yield of 78% in proportion to the dry cell weight at 60 h was observed.

Characterization of the PHB Produced from Sucrose and Molasses

FTIR spectroscopy was used to determine the chemical structure of PHB. In this study, a peak at 1715–1720 cm^{-1} was observed,

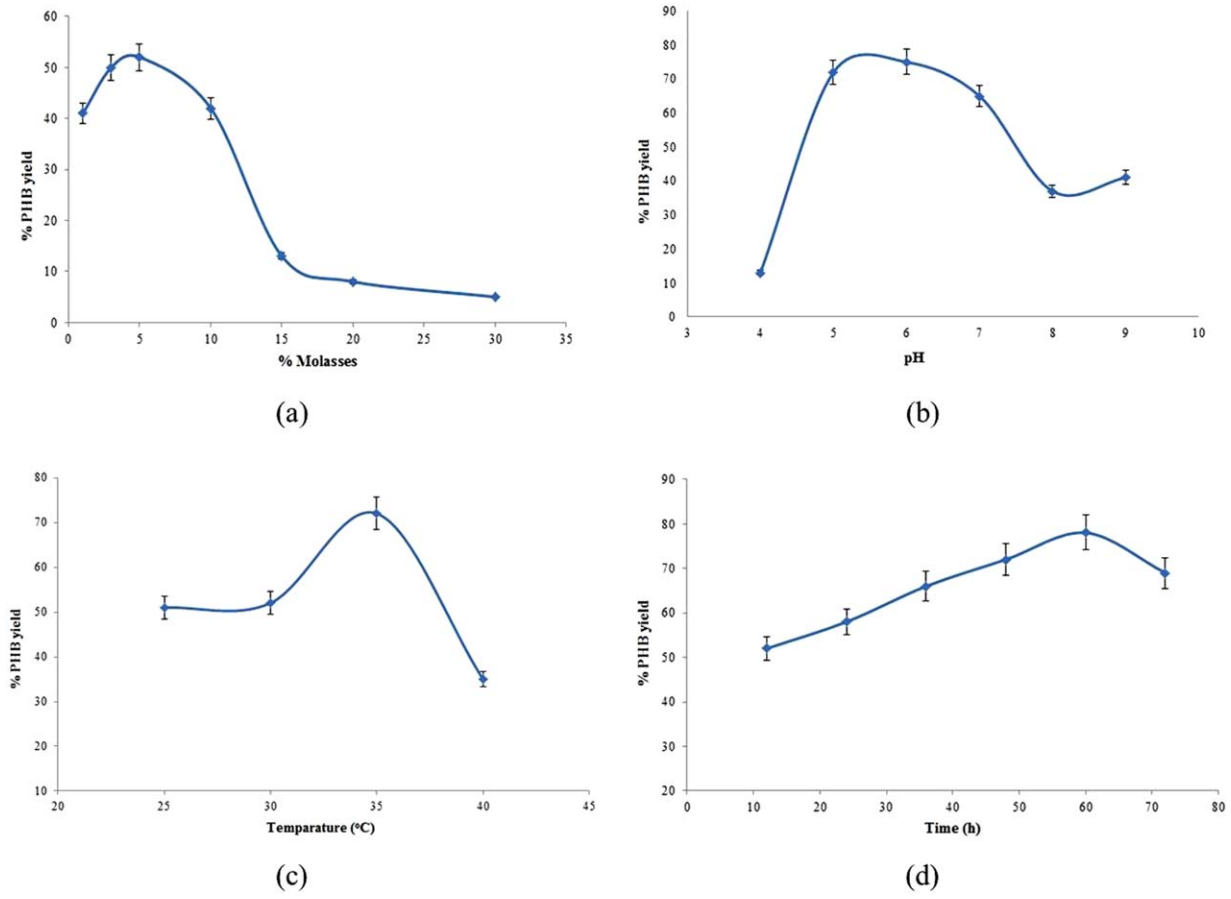

Figure 2. (A) Optimization of the amount of the carbon source, (B) optimization of pH, (C) optimization of the medium temperature, and (D) optimization of the incubation time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

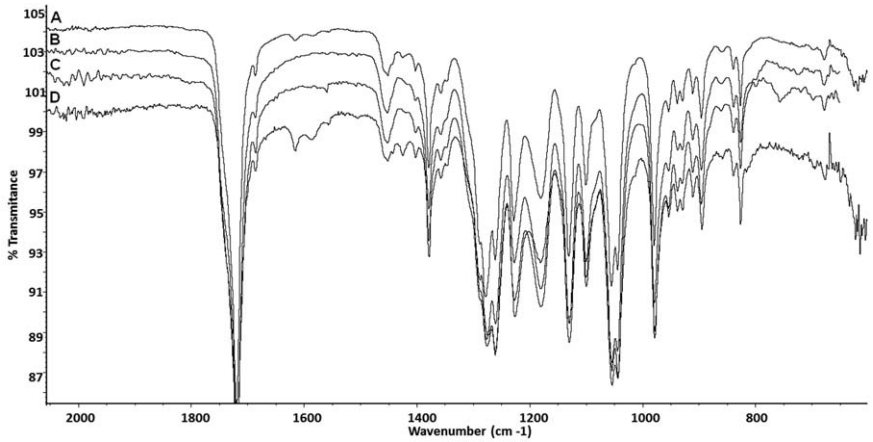

Figure 3. FTIR spectra of the (A) pure commercial PHB provided by Sigma-Aldrich Cheme, (B) PHB synthesized from *A. eutrophus*, (C) PHB synthesized with sucrose as the carbon source, and (D) PHBV synthesized with industrial waste molasses as the carbon source.

and C═O stresses were detected. At the same time, ester linkages in C—O stresses were detected in a 1261–1279-cm^{-1} peak. The characteristic peaks observed in the FTIR spectra are given in Figure 3.

^{1}H-NMR spectroscopy was used to determine the molecular structure of the produced PHB and PHBV. The molecular structure of the polymer produced with sucrose and molasses as a carbon source was compared with those of the pure commercial PHB and PHBV obtained from Sigma-Aldrich Cheme and the PHB synthesized from the commercial strain *A. eutrophus*. In the ^{1}H-NMR spectrum of commercial PHB, a proton peak of CH_3 at 1.27 ppm, a proton peak of CH_2 at 2.49–2.58 ppm, and a proton peak of the CH group at 5.25–5.27 ppm were observed [Figure 4(A)]. In the ^{1}H-NMR spectrum of PHB synthesized from *A. eutrophus* [Figure 4(B)] and with sucrose as carbon source [Figure 4(C)], the peaks and locations coincided exactly with commercial PHB. When the ^{1}H-NMR spectrum of PHBV produced with molasses as the carbon source was examined, we observed that the proton peak of CH_3 belonged to 3HV at 0.88 ppm and the proton peaks of CH_3 belonged to 3HB at 1.27 ppm, the CH_2 peaks at 2.5–2.6 ppm, and the CH peaks at 5.25–5.27 ppm [Figure 4(D)]. These peaks and locations coincided exactly with the commercial PHBV. The copolymer compositions of PHBV synthesized with molasses were calculated with ^{1}H-NMR analysis with eq. (2). The 3HV composition of PHBV was calculated to be 11% 3HV.

The molecular weight of the bioplastics synthesized by the *B. megaterium* strain A1 was determined according to an Ubbelohde capillary viscometer. As a result of the calculations, the viscometric molecular weight of the polymer synthesized with the polymer medium with sucrose as a carbon source was found to be 428 kDa, and the molecular weight of the PHBV synthesized with molasses medium was found to be 491 kDa.

Figure 5 shows the thermograms of the commercial PHB [Figure 5(A)], PHB synthesized from *A. eutrophus* [Figure 5(B)], PHB synthesized with sucrose [Figure 5(C)], PHBV synthesized with molasses [Figure 5(D)] as the carbon source, and commercial PHBV [Figure 5(E)]. The T_m of commercial PHB was observed at 168.05°C, and that of Alcanigenes spp. was observed at 169.35°C. Two T_m values of the PHB with sucrose as the carbon source were observed at 160.84 and 185.26°C. The presence of more than one T_m in PHB with sucrose as the carbon source led us to think that the PHB contained more than one different molecular weight sections. The T_m of the commercial PHBV was at 159.41°C and that with molasses as the carbon source was at 164.77°C.

DISCUSSION

In this study, when the PHB production abilities of 42 isolates, which were obtained as a result of isolation performed in hydrocarbon-contaminated soils, were scanned, we determined that the A1 isolate had a 44% PHB yield. Thirumala et al.[26] made isolations from activated clay, and when their abilities to produce PHB were scanned, they determined that strains 871 and 112A could produce 20–24.2% PHB, respectively. Also, Wu et al.[27] reported that a *Bacillus* sp. JMa5 strain isolated from molasses-contaminated soils could produce 25–35% PHB.[27] When the PHB production ability of the A1-coded strain was compared with the production capability of strains selected as PHB producers in other studies, we observed that this isolate

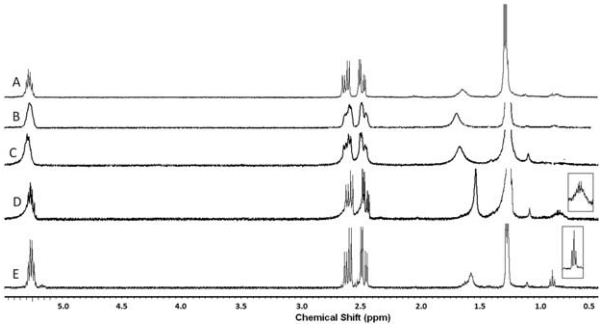

Figure 4. ^{1}H-NMR spectra of the (A) pure commercial PHB provided by Sigma-Aldrich Cheme, (B) PHB synthesized from *A. eutrophus*, (C) PHB synthesized with sucrose as the carbon source, (D) PHBV synthesized with industrial waste molasses as carbon the source, and (E) commercial PHBV provided by Sigma-Aldrich Cheme.

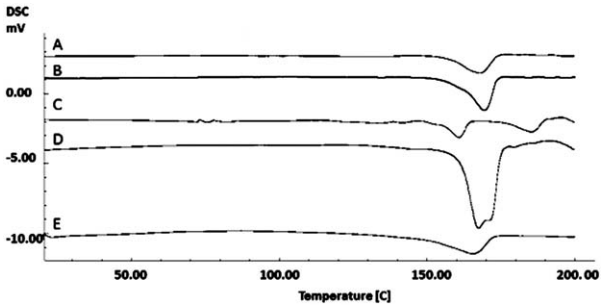

Figure 5. DSC curves of the (A) commercial PHB provided by Sigma-Aldrich Cheme, (B) PHB synthesized from *A. eutrophus*, (C) PHB synthesized with sucrose as the carbon source, (D) PHBV synthesized with molasses as the carbon source, and (E) commercial PHBV provided by Sigma-Aldrich Cheme.

could constitute an important alternative. Therefore, this study was continued with the A1 isolates.

Molecular techniques were used for the species identification of the A1 isolate, and the 16s RNA gene showed that this bacterium was a strain of *B. megaterium* with an accession number of KC579390. There have been studies in which *B. megaterium* was used as a PHB producer in literature. It was reported that the strain *B. megaterium* SW1–2 synthesized 36% of PHB in proportion to the dry cell weight.[28] In a study in which the strain *B. megaterium* BA-019 was used, it was reported that it synthesized 42% PHB in proportion to the dry cell weight under optimum conditions.[29] In this study, the PHB producer strain of *B. megaterium* synthesized PHB at a comparable level with those found in the literature. After this step of the study, it was targeted with an optimization study to increase the PHB yield.

In this study, within which we investigated the effects of both synthetic and industrial waste carbon sources on PHB production, *B. megaterium* strain A1 produced 48% PHB on the nutrient medium containing 3% molasses (Figure 1). When similar studies in the literature were examined, we observed that the yield of PHB synthesized from molasses by *B. megaterium* strain A1 showed comparable results.[27,30,31] In a study carried out with *Bacillus mycoides* RLJ B-017, sucrose, glucose, fructose, lactose, galactose, mannose, rhamnose, xylose, arabinose, ethanol, and methanol were used as carbon sources, and their effects on the PHB production and cell growth were investigated. In conclusion, the best PHB accumulation and cell growth have been observed with sucrose, glucose, and fructose.[32]

On the other hand, in the experiment that was carried out to determine the effect of the carbon source amount on PHB production, a 52% PHB yield was reached in the medium containing 5% molasses (Figure 2). To the best of our knowledge, the results also indicate that the PHB production capacities of some bacteria were similarly induced by molasses. The previous study indicated that the PHB production yields increased with increasing molasses concentration.[30,33]

In one study, it was reported that 46.5–46.3% PHB was obtained in a medium with 2% molasses.[30] In another study, it was reported that *Azotobacter vinelandii* UWD produced 59% PHB in proportion to the dry cell weight in a medium with 5% molasses.[34] The low production cost of petroleum-derived plastics compared to PHB is a significant disadvantage for PHBs being substituted for petroleum-derived plastics. Therefore, the use of more economical carbon resources is crucial for the microbial production of PHB in terms of reducing the costs. In our study, the ability of the *B. megaterium* strain A1 to use industrial waste molasses and to increase the synthesis of PHB in proportion to the dry cell weight had an important effect on the cost of the synthesized PHB.

In the study that was carried out to determinate the effect of the pH on PHB production, we observed that the *B. megaterium* strain A1 produced PHB with a 75% yield in a medium with molasses at pH 6. The PHB production was generally high at neutral levels in the study in which the effect of the pH was investigated (Figure 2). It was reported that the maximum PHB yield was observed at pH 7 in the culture of *Bacillus subtilis*.[35] In a similar study, the optimum pH value in PHB production was found as 6.5.[36] It has been reported that the *Bacillus* sp. 87I and *Bacillus* sp. 112A strains synthesized higher levels of PHB in the polymer medium at pH 7.2.[26]

In the study in which we investigated the effect of the incubation temperature on PHB production, the maximum PHB yield was determined at 35°C (Figure 2). It has been reported in the literature that the PHB production with the maximum yield occurred under mesophilic conditions, and the highest PHB yield at these temperatures were significant for industrial applications.[26,35,36]

As shown in Figure 2, a PHB yield of 78% was reached with 60 h as the incubation time. There was a decrease in the PHB production after 60 h of incubation; the study was not continued after 72 h. In a study carried out with *B. megaterium* SRKP-3, it was reported that the optimum incubation time was 36 h.[33] In addition to studies in which the appropriate incubation time for PHB production was 45 h, there are also similar results in the literature.[37–39]

The characterization studies were carried out to determine the physical and chemical structures of the PHBs synthesized with sucrose and molasses as the carbon source by *B. megaterium* strain A1 in the determined optimum conditions.

FTIR spectroscopy was used to determine the chemical structure. As a result of this study, we observed that the biopolymers synthesized with sucrose and molasses as a carbon source coincided with the FTIR spectroscopy analysis done for PHB in literature.[26,40]

The ^{1}H-NMR spectroscopy method was used to determine the molecular structure of the produced PHBs. The results of this study were compared with those of the PHB and PHBV obtained from Sigma-Aldrich and the PHB synthesized by the commercial strain *A. eutrophus*. These analyses led us to the conclusion that the PHB synthesized with sucrose was similar to the PHBs synthesized by Sigma Aldrich and *A. eutrophus* (Figure 4). It was revealed that the PHB synthesized with molasses also had an hydroxyvalerate (HV) copolymer. The 3HV content of the produced PHBV was calculated with ^{1}H-NMR, and a 3HV monomer content of 11% was detected (Figure 4).[23,41] Some organic acids, which were used as carbon sources in 3HV

syntheses, also exist in waste molasses. For example, organic acids [oxalic acid (0.01%), hydroxyglutaric acid, lactic acid (0.5%), saccharinic acids, humic acids and arabic acids, and also slight amounts of formic, acetic, butyric, and propionic valeric acids] were found in molasses.[42] The usage areas of bioplastics with HV increased compared with other polymers because of their flexibility and because they take shape easily. It was a crucial advantage that it was not necessary to add carbon sources, such as valeric acid or propionic acid, to the medium with molasses for the HV synthesis. In the light of this information, we determined that the synthesized PHBs had the same molecular structure as the PHBs obtained from Sigma-Aldrich Cheme and those synthesized by the commercial strain *A. eutrophus*. We determined that the polymer synthesized with sucrose as a carbon source was PHB, and the polymer synthesized with molasses as the carbon source was Poly(hydroxybutyrate-*co*-hydroxyvalerate). The ^{1}H-NMR spectra obtained as a result of this study were consistent with the ^{1}H-NMR spectra obtained from studies in the literature.[26,43]

The molecular weights of PHB and PHBV synthesized with sucrose and molasses as the carbon sources were calculated to be 428 and 498 kDa, respectively. When the synthesized biopolymers were compared, we determined that the reason for the increase in the molecular weight of the PHBV polymer with HV units was the HV unit. It was reported that the molecular weight of the PHB synthesized by the *Bacillus* sp. 871 strain was determined to be 513 kDa and the molecular weight of the PHB synthesized by the *Bacillus* sp. 112A strain was determined to be 521 kDa.[26]

Similar results were observed when the results of the DSC analysis (Figure 5) that was performed to determine the thermal characteristics of the PHB and PHBV synthesized by the *B. megaterium* strain A1 were compared with the literature. It was reported that the T_m of PHB synthesized by the *B. megaterium* BA-019 strain was 174°C.[29] In one study, PHBV with 680-kDa molecular weight and that contained 11% 3HV was used, and the T_m value was found to be 164.7°C.[44] The T_m value of PHBV (containing 14% 3HV and with a molecular weight of 420 kDa) was determined to be 155°C by Buzarovska and Grozdanov.[25] Wang et al.[45] determined that the T_m value of PHBV containing 5.7% 3HV (molecular weight = 252 kDa) was 171.6°C. These studies and our results show that the 3HV monomer included in the structure of PHB decreased the T_m values and also the crystallinity and increased the elasticity of the polymers.[46]

CONCLUSIONS

As a result of this study, an alternative PHB producer with a high PHB yield and that was more advantageous than commercial PHB in terms of physical and chemical characteristics, was isolated. As a result of these analyses, the most arresting point was that the bioplastic synthesized with molasses as a carbon source is Poly(hydroxybutyrate-*co*-hydroxyvalerate) with 11% HV.

We suggest that the *B. megaterium* strain A1 isolated from hydrocarbon-contaminated soil is an alternative PHB and PHBV producer under moderate conditions. The PHB yield reached 78% from 44% after the optimization of the conditions of the medium with the use of molasses as the sole carbon source. Therefore, this study was important because it yielded *B. megaterium* strain A1, which was capable of producing PHB not only from sucrose but also from industrial byproduct carbon source, for instance, molasses. Also, this study demonstrated the ability of this isolated *B. megaterium* strain A1 to produce PHBV from molasses with a white color, which was the same as that produced from sucrose. Generally, when we compared our results with those of other studies on PHB or PHBV production with molasses by other bacteria, we observed a high productivity, greater flexibility, and lower caramelization with the use of a high sugar content of molasses. For pilot and/or large-scale productions, the inclusion of molasses as a sole-carbon-source medium will be very functional, and also, this process can act as a key for the use of byproducts of sugar factories in an efficient and low-cost way.

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