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〈研究論文(學術)〉

Microbial Poly-β-hydroxybutyrate의 구조특성

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Characterization of microbial poly-β-hydroxybutyrate

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Abstract—Poly-β-hydroxybutyrate(PHB) was biosynthesized using *Alcaligenes* sp. FL—027. *Alcaligenes* sp. FL—027 was cultivated by fed-batch methods, in order to promote cell growth and PHB accumulation with carbon source. The cells were first grown at 30℃ on the fermentor. The structure of biosynthesized PHB is investigated by the NMR, IR. The crystalline portions were identified through the use of DSC and X-ray diffractometer. The melting point was about 160℃ and the diffraction peaks of (020) and (110) were shown at 13° and 17°, respectively.

1. INTRODUCTION

Plastic industry is developed in almost half of century by merits of stability during long periods and of easy property of casting. The production effected to life is used in a variety of regions that are medical service, leisure, prevention of environment, building construction and several industries and so on.

But the rapidly expanding production and the use of plastic materials create massive problems in the area of waste disposal. Developing biodegradable plastics in the key to solving the waste disposal problem, and developing plastics that do not pollute the global environment have become an extremely important objective.

The microbial poly(3-hydroxyalkanoate) family of polyester is thermoplastic with biodegradable and biocompatible properties and produced from various carbon substrates by microorganisms. These microbial polyesters have recently attracted industrial attention as large-scale biotechnological products. The biosynthesis, structure, and properties of poly(hydroxyalkanoates) have been extensively investigated at both academic and industrial research centers. Today research and development on microbial polyesters are rapidly expanding in the biological polymer science[1-4].

In this paper, we have studied the effects of various carbon and nitrogen sources that will each effect both the cell growth and PHB accumulation to varying degrees. The microorganism used in this study was *Alcaligenes* sp. FL-027. The microorganism was cultivated to promote

cell growth and PHB accumulation from carbon sources by means of fed-batch culture technique on optimum condition. The structure, thermal property and crystallinity of PHB biosynthesized by *Alcaligenes* sp. FL-027 were investigated by NMR, IR, DSC, X-ray diffractometer, and free volume contents.

2. EXPERIMENTAL

2-1. Bacterial strain

Alcaligenes sp. FL-027 was used in this study. *Alcaligenes* sp. FL-027 cell was grown at 30℃ and pH 7 for 24hrs as 300rpm of agitation speed in 5 l fermentor(B.Braun, Biostat® E) containing 2 l of culture ground. The culture contained 8g of fructose and 2g of $(NH_4)_2SO_4$ per liter of distilled water.

2-2. Growth conditions and PHB synthesis

Alcaligenes sp. FL-027 was cultivated by fed-batch cultivation[5-7], in order to promote cell growth and PHB accumulation with carbon source. The cell was first grown at 30℃ on a fermentor. Initial medium contained 16g of fructose, 6g of $(NH_4)_2SO_4$ per 2 liter of distilled water. In order to promote PHB synthesis, high concentration culture ground is added every 1 or 2hrs for cultivating. Table 1 summarized the condition of fed-batch culture used in this study.

Table 1. Condition for fed-batch culture

	Initial medium	Primary feeding	Secondary feeding
Fructose(g)	16	200	50
$(NH_4)_2SO_4$(g)	6	74.6	1.66
Volume(ml)	2000	300	200
pH	7.0	7.0	6.5
Agitation speed(rpm)	300	300	300

2-3. Quantity and Purification of PHB

2-3-1. Quantitative analysis of PHB

The samples were harvested by centrifugation in screw-cap tube containing 5~10ml culture solution as pre-cure. After eliminated above phase solution, then the samples put on 2ml of methanol(3% H_2SO_4) and 2ml of chloroform at 100℃ for 3.5 hrs. And then the solution strongly shaked for 10 min with 1ml distilled water was cooled at room temperature. Among to two phases eliminated above phase after putting with several minutes, quantitative analysis of PHB was carried out with gas chromatography (Hewlett packard).

2-3-2. Purification of PHB

PHB solution was extracted several times with chloroform after colony lysis. Then PHB was selectively precipitated with 1~2 times of methanol in chloroform. PHB was harvested by filter paper(Whatman filter paper No.1) and was repeated this purification[8].

2-4. Measurements

2-4-1. ^1H NMR measurements

^1H NMR spectra of PHB in $CDCl_3$ solution were carried out on a BRUKER AM 300 spectrometer. The 300MHz ^1H NMR spectra were observed at 27℃ in $CDCl_3$ solution of PHB(5mg · cm^{-3}) with 30-s pulse repetation, and 16k data points[9-12].

2-4-2. ^{13}C NMR measurements

^{13}C NMR spectra of PHB in $CDCl_3$ solution were carried out on a BRUKER AM 300 spectrometer. The 75MHz ^{13}C NMR spectra were observed at 27℃ in $CDCl_3$ solution of PHB(25mg · cm^{-3}) with 30-s pulse repetation, and 16k data points[9-12].

2-4-3. DSC measurements

The DSC thermogram of PHB were carried out on a DELTA SERIES DSC7. The heating

rate of 10℃min was used for determination of the melting point.

2-4-4. IR measurements

The IR spectra were taken by KBr pellet method with disperse IR. The Transmittance of characteristic bands were obtained by peak heights of spectra.

2-4-5. X-ray measurements

Wide angle X-ray diffraction measurements were carried out on a Rigaku D/max-Ⅲ-A. CuK_α radiation(λ=1.5418 Å) was used as the source. The X-ray diffraction patterns of PHB were recorded at room temperature in the range $2\theta=5\sim40^\circ$ at scan speed 2°/min.

2-4-6. Density measurements

The film was prepared by 1g of PHB powder dissolved in 10ml of dichloromethane. And then solvent was evaporated on the glass plate (7×7 cm^2) at room temperature in hood. The film was washed for 5hrs with alcohol by Soxhlet's extractor and was dried for 12hrs by vacuum desiccator.

The density of PHB was measured by density gradient tube. It was composed of carbon tetrachloride(d=1.59) and n-hepthane(d=0.68) for density range from 1.15g/cm^3 to 1.38 g/cm^3. The tube was maintained for 15days at 25℃ and for 15days by means of a water jacket and calibrated with standard floats.

3. RESULTS AND DISCUSSION

3-1. Structure of PHB

Fig. 1 shows the 300MHz ^1H NMR spectra of PHB sample at 27℃ in $CDCl_3$ solution. The assignments of signals have been reported each proton resonance. The conformational structure around the CH_2-CH bond in solution can be determined by the analysis of the methylene proton resonance.

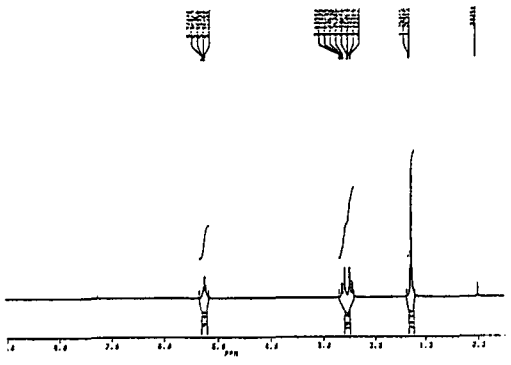


Fig. 1. 300MHz ^1H NMR spectra of PHB at 27℃. Chemical shifts are in ppm from $CDCl_3$.

In addition to the shielding, electrons produce additional effects due to the magnetic moments of neighboring nuclei. Since this part of the coupling occurs via chemical bond, it is independent of spatial orientation. This is interaction survives the effect of molecular motions.

We expect that the methylene protons will give rise to a resonance pattern having a different chemical shift than the methyl protons. If methyl proton is coupled with the methylene proton, we must view with some detail the effect of methylene proton on the methyl proton. One of the protons on the methylene proton can be oriented either with against the field. Therefore any given methylene proton can display three possible total orientations of their spins, as shown Fig. 2. In one case they are both oriented with the field, in two cases they are oriented in opposite directions, and in one case they are both against the field.

Thus the methyl protons, interacting with the methylene protons both oriented with the field, would come into resonance at a slightly lower field than all others. If one spin were oriented in each direction, then the small magnetic field

produced would be canceled by the other, so that there would be a net contribution of zero to the applied magnetic field.

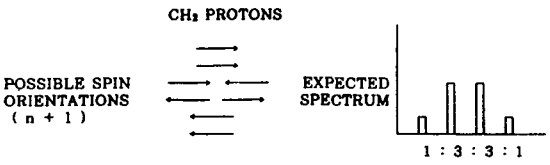


Fig. 2. Spin-Spin coupling for poly-β-hydroxybutyrate.

The conformational structures generated by rotation the CH_2-CH bonds of methylene proton resonance in the 300 MHz ^1H NMR spectra of PHB in chloroform. The $_2C$ methylene proton resonance of PHB at 2.42~2.63 ppm was associated with the $_3C$ methine proton(H_x) and was analyzed as ABX type with vicinal coupling of HA and HB protons[13]. Fig. 3 shows resonance for PHB.

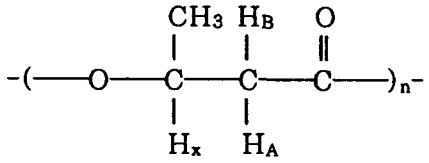


Fig. 3. ABX type with a vicinal coupling of H_A and H_B protons.

The observed vicinal coupling constants J_{AX} and J_{BX} of $_2C$ proton resonance in PHB, indicating that the hydroxybutyrate(HB) units of PHB in $CDCl_3$ solution have identical conformational structure for the CH_2-CH bonds of the backbone.

The observed vicinal couplings are averaged over the three possible conformers ; trans(T), gauche(G), and another gauche($\bar{G}$), as shown by Newman projections of the three rotational isometric states.

Fig. 4 shows the 75MHz ^{13}C NMR spectra of PHB, together with chemical shift assignment for each carbon resonance. The three ^{13}C resonance's at 19.742~67.647 ppm could be assigned to specific carbon species in 3HB units.

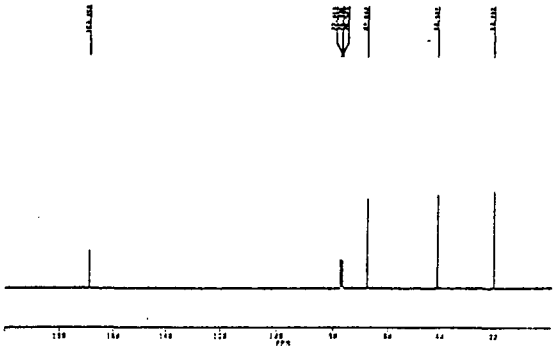


Fig. 4. 75MHz ^{13}C NMR spectra of PHB at 27℃. Chemical shifts are in ppm from $CDCl_3$.

The extended spectra of butyrate from PHB exhibited a specific enhancement in the intensity of CH_2(42.53), CH(67.64), CH_3(19.24), carbonyl carbon resonance at 169.35ppm are shown Fig. 4. These results indicate that the ^{13}C-labeled carbonyl carbon of butyrate is selectivity incorporated in the carbonyl carbon of PHB without randomizing to other carbons. On the other hands, the ^{13}C-labeled carbonyl carbon is selectively incorporated in the carbonyl and methine carbons of PHB.

The IR spectra of PHB are shown in Fig. 5. The characteristic absorptions of PHB include a band at 1730cm^{-1} representing -COO stretching, an extra strong band at 2960cm^{-1} which corresponds to -CH stretching.

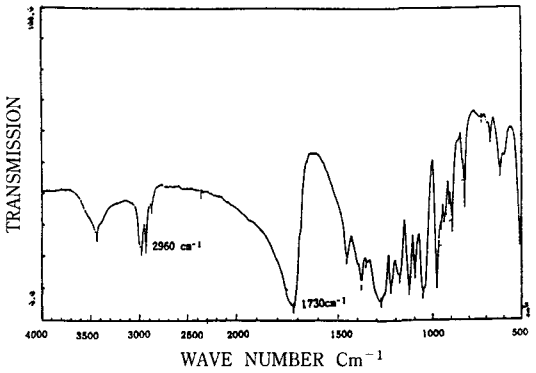


Fig. 5. IR spectrum of PHB by KBr pellet method.

3-2. Thermal property

Fig. 6 shows the DSC thermogram of PHB. The melting point of PHB estimated with the thermal data. This predicts thermoplastic material with low melting point about 160℃. In the figure, it can be considered that melting point of PHB is attributed to melting of crystalline portion of PHB.

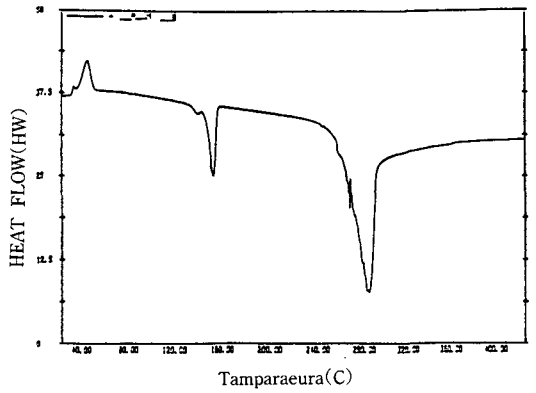


Fig. 6. DSC thermogram of PHB.

3-3. Crystallinity of PHB

Fig. 7 shows the X-ray diffraction profile of PHB. In the figure, it is considered that PHB has crystalline region owing to shown sharp peaks around 13°, 17°, 20°, 25° and 30° shown in fig. 7.

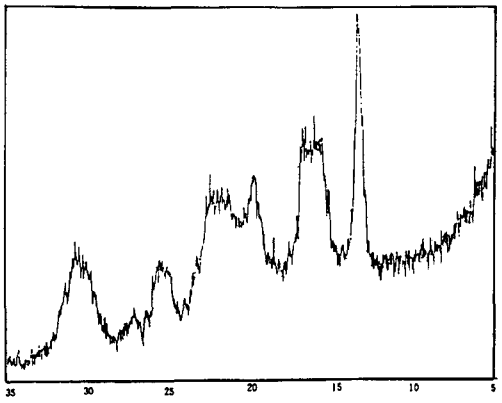


Fig. 7. X-ray diffraction profile of PHB.

The unit cell of crystal phases PHB was orthorhombic[14], the (020) and (110) d-spacings of PHB were calculated from x-ray diffraction curve by Bragg's equation.

Bragg's equation

$$2d \cdot \sin\theta = \lambda$$

where, d : d spacing
θ : Bragg's angle
λ : x-ray wavelength(1.541 Å)

The (020), (110) d-spacings are calculated d_{020} =6.46 Å, d_{110}=5.44 Å. Cornibert[15] calculated d-spacings of untreated PHB as a=5.76 Å, b=13.20 Å, c=5.96 Å (fiber repeat). In according to, we can predict that biosynthesized PHB has crystalline regions.

3-4. Free volume content

Generally the free volume theory predicts that quantities related to mobility very exponentially with the reciprocal of the free volume. A major imitation of free volume theories to the quantitative use is the lack of a mean to measure experimentally. In order to calculate the free volume of a polymer from simple density measurement,

the 0 K occupied volume of the various atoms or molecular groups in the polymer repeat unit be estimated from the following simplistic definition of specific free volumes(V_f) ;

$$V_f = V - V_o$$

where, V : experimentally measured specific volume

V_o : specific volume at the absolute zero temperature

The several methods are available for obtaining V_o. The group contribution method of Bondi[16] is used in this study. It is based primarily on X-ray diffraction data which are used to estimate van der Waal's volumes and radii for a variety of molecular groups.

The suggested relation between the occupied volume at 0 K, V_o, and the van der Waal's volume, V_w, is

$$V_o = 1.3\ V_w$$

The free volumes obtained from the above calculations are estimates based on certain assumptions that may limit their validity. However the Bondi's method is widely used elsewhere since it is simple and direct.

The results calculated by group contribution methods ;

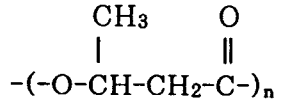
density = 1.263 g/cm^3
free volume = $V - V_o$ = 0.2037 cm^3/g
fractional free volume = $(V - V_o)/V$ = 0.2573 cm^3/g

The free volume of the pure component is the result to be expected if simple volume additivity occurs, whereas the deviation from this ideality is the excess volume of sorption. As the free volume means the mobility of molecules, the larger free volume of PHB than PAN(d=1.168, free volume=0.0784, fractional free volume=0.0916) indicates that PHB has less packing structure and better flexibility than PAN.

4. CONCLUSIONS

Poly-β-hydroxybutyrate(PHB) was biosynthesized using *Alcaligenes* sp. FL-027. The structure and thermal property were identified through the use of spectroscopies of NMR, IR, and DSC.

From all spectral data of the PHB, the molecular structure is predicted as the following ;



It was found that biosynthesized PHB is a melt processible thermoplastic material with low melting temperature about 160℃.

PHB has crystalline region, because X-ray profile shows sharp peaks around 13° and 17°. The d-spacings of (020) and (110) planes are d_{020}=6.46 Å and d_{110}=5.44 Å, respectively.

The free volume content(0.2037 cm^3/g) was calculated from measured density. So, it can be predicted that PHB is easily biodegradable and flexible because of low packed structure.

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