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Biosynthesis and characterization of poly(β-hydroxybutyrate) produced by *Bacillus circulans*

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Summary.
The biosynthesis of poly (β-hydroxybutyrate) (PHB) by *Bacillus circulans* was carried out through cultivation in nutrient broth in a single step. $\bar{M}v$ of polymer fractions soluble in chloroform were close to 2 x 10^5. IR, ^1H and ^{13}C NMR spectra of the isolated PHB were in agreement with the assumed structure of this poly-β-hydroxyester. NMR spectroscopy pointed out a high percentage of isotactic polymer, while differential scanning calorimetry showed that the PHB was crystalline.

Introduction

It is well known that poly (β-hydroxybutyrate) (PHB), a linear polymer on the poly(β-hydroxyalcanoate) (PHA) family, appears as reserve material in a wide variety of bacterial species functioning as carbon and energy storage for many microorganisms (1-4). Several authors have carried out studies on the microbial production and characterization of PHB and copolymers that contains β-hydroxybutyrate (HB) and β-hydroxyvalerate (HV) (5). In general, the microbial production of PHA is carried out in two steps. The first one consists in the growth of microbial cells in the presence of all necessary elements (C, N, P); while in the second step, bacterial cells grow under limiting conditions and with an excess of carbon substrate (2).
We have obtained PHB using *Bacillus circulans*, grown under improved culture conditions and characterized the isolated polymer through ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and differential scanning calorimetry.

Experimental part

Biosynthesis of PHB.

The cells were grown under aerobic conditions. The microbial biomass was obtained by culture for 48 hours, using carbon, nitrogen and all microelements source, supplied in a nutrient broth (Oxoid).
The PHB content was determined qualitatively by observing the intracellular granules with sudan black lypophilic staining. In other experiments, after 48 hours of cultivation, an excess of carbon substrate was added and an additional 48 hours of growth was allowed. The carbon substrates were: a) molasses, b) glucose or c) glucose + butanol.

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PHB isolation.
The cells were separated by centrifugation (20.000 g x 20 min.), washed with 10 mM Tris-0.9% NaCl buffer solution (pH = 7) and resuspended in 0.5% NaClO solution (added 50 mL of NaClO solution to 200 mL of initial culture). This suspension was stored for one hour, at 37°C and after centrifugation (4000 g by 10 min.), the precipitate was washed with water, acetone and ethanol consecutively. Finally, the polymer was dissolved in chloroform, by refluxing for 6-8 hours. The solvent was evaporated and a PHB film was obtained.

NMR spectroscopy.
The NMR measurements were carried out at 300 K using a Brucker AC 250F spectrometer at 250 MHz (^{1}H) and 62.9 MHz (^{13}C). Samples were analyzed in 5 mm sample tubes in chloroform-d. The spectra were referenced to internal TMS.

IR spectroscopy.
IR spectra were recorded on Specord 75 IR Carl Zeiss Jena.

Thermal Analysis.
The glass transition (Tg), melting temperature (Tm), and the heat of crystallization and fusion (DHc and DHm) were measured using a Mettler TG 50, DSC 30 instrument.
In the gravimetric analysis, the weight of each sample was typically 12-15 mg and was heated at the rate of 20°C/min. In the DSC analysis, the sample (5-10 mg) was heated at the rate of 10°C/min from 30 to 210°C, quickly cooled, and then scanned a second time using the same heating rate and temperature range as in the first scan.

Molecular weight measurements.
Molecular weights were determined by viscometry with a Lauda automatic viscometer (t = 0.01 s) in chloroform solution at 30°C. The limiting viscosity number [η] was calculated by the Huggins and Kramer equation (6) and the molecular weights by the Mark-Houwink equation.

Results and discussion.

PHB production.
To achieve good PHB yields it is essential to obtain both high cell yields and high polymer content. Table 1 exhibits the results of two variants of cells growth. In the first process,

TABLE 1. PHB production by Bacillus circulans

Process	Cell yields[(a)] (g/L)	Polymer content (%)
One step	14.29	>50
Two steps		
a) Molasses	1.88	<5
b) Glucose	1.86	<5
c) Glucose + Butanol	1.45	< 10

(a) Wet weight.

TABLE 2. Molecular weight of PHB fractions soluble in chloroform.

Fraction	[η]	$\bar{M}v^{(a)}$	% in the sample
Sol. at 20°C	1.55	190550	39
Sol. at 30°C	1.804	231490	46

(a) Constant used: $K = 11.8 \times 10^{-5}$; $a = 0.78$ (9).

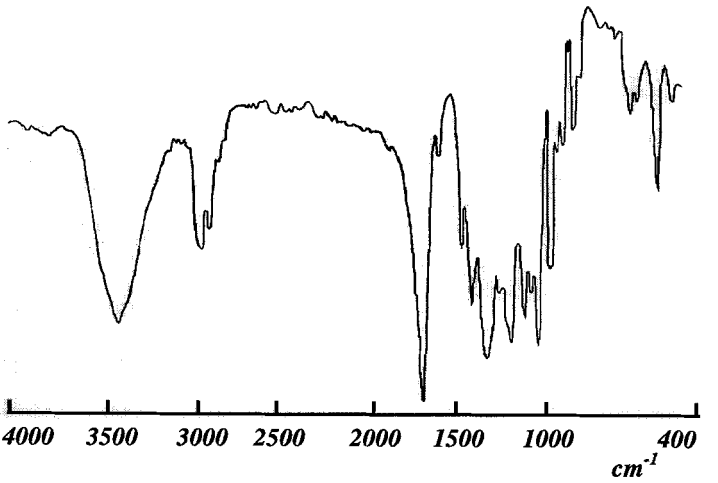


Figure 1. IR spectrum of PHB obtained by *Bacillus circulans*.

These results point out that *Bacillus circulans* produces PHB in one step, without stress conditions. Similar behavior is observed with the *Azotobacter vinelandis* UWD mutant (1), which accumulates polymer in growing, oxygenated, nutrient-sufficient conditions because it is deficient in NADH oxidase and responds as if it were oxygen limited.
At 30°C, 15% of a polymer sample was insoluble in chloroform. The soluble portion was separated in two fractions, one soluble at 20°C and the other at 30°C. The $\bar{M}v$ of both fractions (see Table 2) were close to 2×10^5.

Spectroscopic characterization.

IR.
The most important bands of the IR spectrum for the ester are observed in Figure 1. An absorption band at about 1730 cm^{-1} is characteristic of a carbonyl group and a band at about 1280-1053 cm^{-1} characterizes the valence vibration of the carboxyl group.

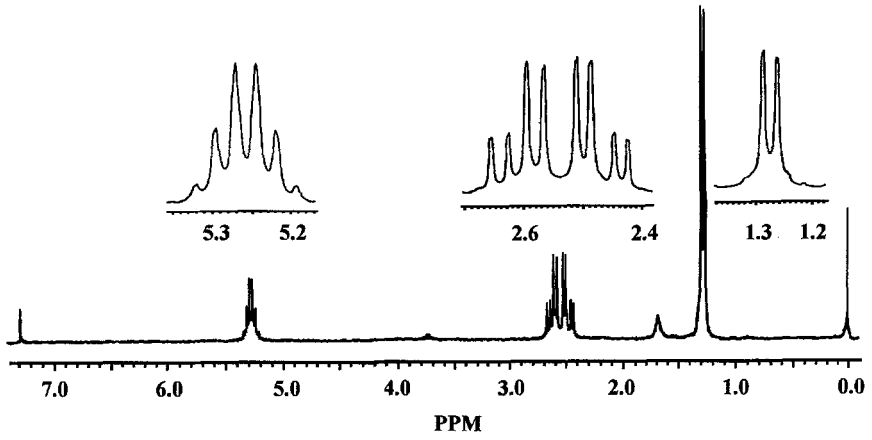


Figure 2. ^{1}H NMR spectrum of PHB obtained by *Bacillus circulans*.

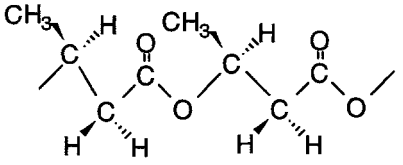
NMR.

The 250 MHz ^{1}H NMR spectrum of PHB in chloroform-d solution is shown in Figure 2. The methine proton attached to the asymmetric carbon is a multiplet at 5.26 ppm, the methylene is a multiplet at 2.61-2.47 ppm and the CH_3 signal is a simple doublet at 1.27 ppm. The signal at 1.6 ppm is water. It was identified by adding a drop of deutered water to the polymer solution and repeating the ^{1}H NMR spectrum after 48 hours; then, the signal at 1.6 ppm disappeared.

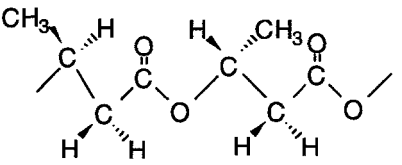
The 62.9 MHz 13C NMR spectrum of PHB in chloroform-d solution is shown in Figure 3. Assignment of the chemical shifts for carbon is explained by inductive effect: carboxyl group, 169.19 ppm; methine group, 67.64 ppm; methylene group, 40.79 ppm; and CH_3 group, 19.79 ppm; which are in agreement with the calculated chemical shifts.

It is known that bacterial PHB is highly stereoregular (7), in this work, we have analyzed the structure by configurational diads.

In general, the configurational diads have the form:



isotactic



syndiotactic

The protons of the CH_2 group, in isotactic form, are not equivalent because they have different chemical environments. They are an A-B system in the ^{1}H NMR spectrum. The signal multiplicity by each proton will be four and eight signals will be obtained due to proton coupling.

The protons of the CH_2 group, in syndiotactic form, are equivalent because they have equivalent chemical environments. They are an A_2 system in the ^1H NMR spectrum. The signal multiplicity will be one and coupling with the proton of the CH group will show two signals.

The signal for the CH_2 protons of the obtained polymer is an octect in the ^1H NMR spectrum (Figure 2) showing a high percentage of isotactic form in our polymer.

Expansion of the carbonyl carbon region in the ^{13}C NMR spectrum (Figure 3) shows a unique signal confirming the isotactic structure in the polymer.

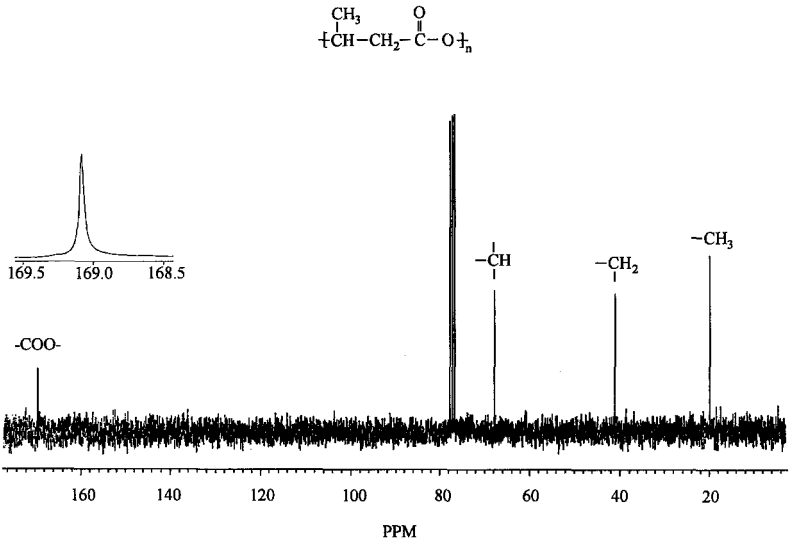


Figure 3. ^{13}C NMR spectrum of PHB obtained by *Bacillus circulans.*

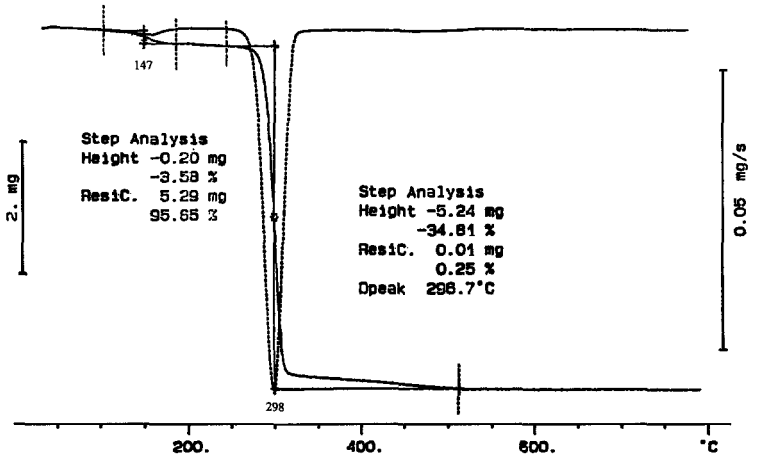


Figure 4. TGA of PHB obtained by *Bacillus circulans.*

Thermal Analysis.

The TGA thermogram is shown in Figure 4. Two decomposition temperatures are evident at 147°C and 298°C with the second accounting for 95% of the weight loss; to reduce this loss calorimetric analysis was carried out at 210°C.

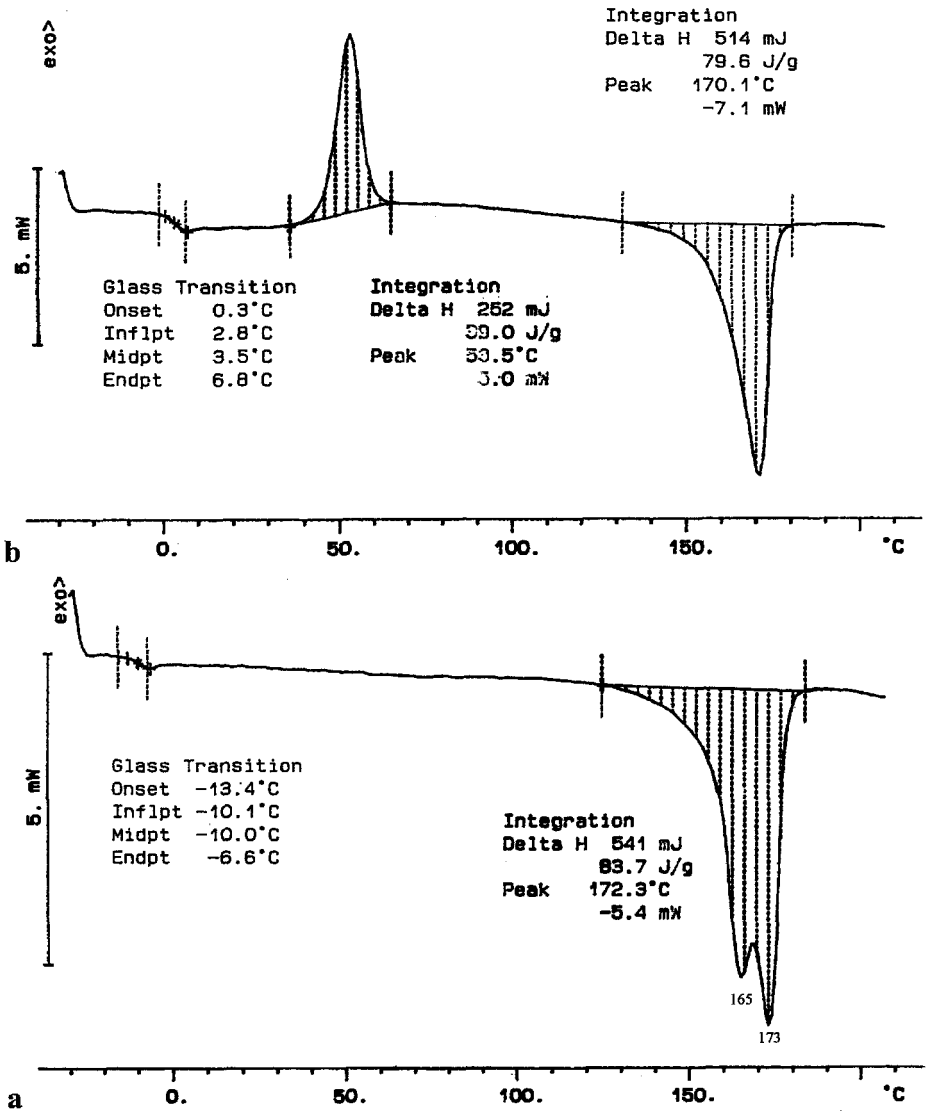


Figure 5. DSC of PHB obtained by *Bacillus circulans.*

a) First scan.

b) Second scan after quickly frozen at nitrogen liquid temperature.

The DSC thermograms for the first heating scan (Figure 5a) exhibit a sharp melting endotherm, characterized by a double peak at 165 and 173°C. The areas under the curve correspond to the enthalpy of fusion, DHm = 83.7 J/g. The absence of a marked glass transition is consistent with the high degree of crystallinity of this sample (8).
After the first heating scan, the sample was quickly frozen at liquid nitrogen temperature then reheated to 210°C. The DSC thermograms (Figure 5b) exhibit a glass transition near 3.5°C, a sharp melting endotherm at 170°C corresponding DH = 79.6 J/g, and a sharp crystallization exotherm at 53°C with DH = 39 J/g. Thus, the sample has a high percentage of crystallinity with a fusion temperature at 173°C. The disappearance of the signal at 165°C after the first scan could be explained by secondary crystallization.

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