Microbial Polyester Synthesis: Effects of Poly(ethylene glycol) on Product Composition, Repeat Unit Sequence, and End Group Structure†

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ABSTRACT: Poly(ethylene glycol) of M_n 200 g/mol (PEG-200) was added to cultivation media of *Alcaligines eutrophus* during polymer formation, and the carbon source used was 4-hydroxybutyrate. The addition of PEG-200 from 0 to 2 wt % resulted in increased 4-hydroxybutyrate (4HB) incorporation from 66 to 86 mol % into the poly(3-hydroxyalkanoate) (PHA), formed. When 4% PEG was used, the mole percent of 4HB in PHA decreased to 64 mol %. Thus, PEG in cultivation media resulted in polyester compositional changes. An unexpectedly large amount of the dry cell weight (∼10%) was found to be PEG-200 for cultivation media amended with 4% PEG-200. Analysis of this product by gel permeation chromatography (GPC) showed it to be complex, having multiple peaks. The repeat unit sequence distribution determined by 75 MHz ^{13}C NMR spectroscopy indicated that the addition of PEG-200 to media resulted in the formation of a product with predominantly 3-hydroxybutyrate (3HB)−3HB and 4HB−4HB diads instead of random copolyesters which are formed in the absence of PEG addition to media. Two dimensional homonuclear (^{1}H) correlated NMR analysis along with a number of control experiments indicates that PEG was covalently linked at the carboxy terminal position of PHA chains, forming natural−synthetic diblocks. Fractionation of this product into acetone soluble (AS, 84 mol % 4HB, M_n = 37 400 g/mol) and insoluble (AIS, 95 mol % 3HB, M_n = 130 000 g/mol) fractions was carried out which proved that the product formed is a mixture of polyesters differing in composition and molecular weight. Analysis of the unfractionated product as well as AS and AIS fractions by differential scanning calorimetry supported the above conclusions.

Introduction

Poly(hydroxyalkanoates) (PHAs) are a series of optically active polyesters made by numerous microorganisms. A number of reviews on PHAs have been published[1−4] that describe biochemical aspects of polymer formation, structural variability, and properties. Since microbial polyesters are synthesized in aqueous media from renewable resources to form thermoplastic water insoluble products, the process for polymer synthesis is extremely attractive as an "environmentally friendly" preparative route. Also, microbial polyesters are biodegradable[5] so that they may be disposed as part of the biowaste (yard, food, and other organic waste) fraction of the municipal solid waste stream.[6−11]

Poly(3-hydroxybutyric acid) (P3HB) was the first identified member of the PHA family.[12−14] A problem associated with P3HB is that melt crystallized and solution cast P3HB films show brittle behavior which increases upon aging at room temperature.[15−18] Motivated by an interest to obtain PHAs with improved physicomechanical properties as well as through a fundamental desire to define the flexibility of the microbial biosynthetic machinery,[3,4] over 50 different structural repeat unit types have been incorporated into PHAs so that a large range of homo- and copolyesters can now be produced. Some selected exampes are as follows: poly[3-hydroxybutyrate-3-hydroxyvalerate-*co*-3-hydroxyhexanoate] (P[3HB-*co*-3HV-*co*-3HH]),[19] P(3HB-*co*-3HH),[20,21] and P3HV.[22] The copolyester of P(3HB-*co*-3HV) is produced on a 660 000 lb/year scale by Zeneca BioProducts.[23] Medium side chain 3-hydroxyalkanoates that contain *n*-alkyl side groups with lengths generally from propyl to nonyl have been the subject of much study of late.[1−4] These PHAs have been prepared with interesting functional side chain substituents such as phenyl[24,25] and cyanophenoxy groups.[26,27] Also, a number of PHAs have been reported that contain 4-hydroxbutyrate (4HB) repeat units. Important examples include poly(3HB-*co*-4HB)[28−31] and terpolyesters of 3HB, 3HV, and 4HB.[32] It has been shown by ^{13}C NMR analyses that P(3HB-*co*-4HB) approximate random copolyesters.[31,33,34] The incorporation of 4HB repeat units into 3HB containing chains was found useful as a means for obtaining materials that have improved percent elongation (5−444%) for copolyesters containing 0 and 16 mol % 4HB, respectively.[30] Also, the formation of the P4HB homopolymer by a mutant strain of *Alcaligenes eutrophus* (*A. eutrophus*) has been reported which amounted to 10% (w/w) of the cellular dry matter.[35]

Poly(ethylene glycol) (PEG) is a neutral water soluble polyether with extraordinary biological properties. PEG is relatively nontoxic to cellular systems, which is illustrated by its use for organ preservation.[36] PEG is also used in processes such as protein purification by "phase partitioning" and protein modification for solubilization where the proteins remain active in the presence of PEG.[37,38] Interestingly, large quantities of PEG are absorbed by membranes,[39,40] and PEG is known to associate with membrane phospholipid head

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groups.[39] Also, alcohols are well-known to cause changes in membrane lipid composition, which results in increased permeability to ions and small metabolites and increased membrane fluidity.[41] Furthermore, it was shown that PEG in cultivation media results in an osmotic stress that is tolerated by the accumulation in cells of a solute such as potassium or calcium ions.[42]

Since PEG has such interesting interactions with cell membranes, proteins, and metabolism, we undertook investigations to determine whether PEG would interact with PHA biosynthetic systems to alter the course of the polymerizations. In other words, our interest was to investigate whether the addition of PEG to fermentation media would offer unusual opportunities for PHA structural control. Effective demonstration of this idea might be termed "PEG modulated fermentation". PEG with number average molecular weight (M_n) 200 g/mol (PEG-200) was added in an amount up to 4% (w/v) to cultivations of *A. eutrophus* during the polymer production stage. The effects of PEG-200 on the conversion by *A. eutrophus* of the carbon source 4-hydroxybutyrate to polyester was studied. Product molecular weight averages and dispersity were investigated by gel permeation chromatography (GPC). The repeat unit composition and incorporation of PEG-200 in various product fractions was investigated by 1- and 2-D ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Polymer repeat unit sequence distribution was studied by ^{13}C NMR spectroscopy. Fractionation by differential solubilities in acetone was used to investigate product heterogeneity. Also, information on thermal transitions of product and product fractions was obtained by differential scanning calorimetry (DSC).

Experimental Section

Materials. 4-Hydroxybutyric acid sodium salt and PEG-200 (200 g/mol) were purchased from Aldrich and used as received. The PEG M_n was confirmed by using ^{1}H NMR end group analysis and was found to be 194 g/mol. Peptone, yeast extract, and meat extract were from Difco. All inorganic media components were purchased from Sigma.

Bacterial Strain Preservation[43] and Innoculum Preparation. *A. eutrophus* (ATCC 17699) was used in this study. This strain was first grown under aerobic conditions[44] at 30 °C for 14 h, and then the culture was diluted with 2 parts of 20% glycerol and transferred to 1 mL cryogenic vials. The vial contents were frozen in a dry ice−ethanol bath, and then the vials were stored in liquid nitrogen. The cells contained in the vials were used as the inoculum for the two-stage fermentation reactions described below.

Fermentations. (a) Cultivations of 100 mL (Cultivation Condition A). A nutrient rich medium (100 mL, the medium composition is described in ref 29) was prepared and autoclaved to sterilize. It was inoculated with 0.1 mL cells from a thawed cryovial. Growth of *A. eutrophus* was carried out in 500 mL baffled Erlenmeyer flasks at 30 °C, and 250 rpm, for 24 h. The cells were harvested by centrifugation and washed with a sterile Na_2HPO_4−NaH_2PO_4 buffer solution at pH 7.0. Typically, the cell dry weight of these first stage cultivations was 3.5 g/L. The washed cells were then transferred under aseptic conditions into 100 mL of a sterile filtered nitrogen free medium which contained 1.51 g/L Na_2HPO_4, 2.65 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 1.0 mL/L Microelement solution,[29] 4-hydroxybutyric acid (1.5 g), and either 0, 1, 2, or 4% (w/v) PEG-200. Polymer production was then carried out by cultivation of *A. eutrophus* in the above media, using a 500 mL Erlenmeyer flask at 30 °C, and 250 rpm, for 48 h. The cells were then separated by centrifugation, washed with about 10 mL of water/(g of wet cells) and lyophilized.

(b) Cultivations of 500 mL (Cultivation Condition B). Increased PHA from media amended with 4% PEG needed for fractionation and subsequent analysis (see below) was obtained as described above by the two-stage method but with use of 2800 mL Erlenmeyer flasks and 500 mL cultivation volumes.

Polymer Isolation. The intracellular PHAs formed were extracted from cells by stirring a suspension of the lyophilized cells (about 0.5 g) for 48 h in chloroform (80 mL) at room temperature. The insoluble cellular material was removed by filtration, and then the solvent was evaporated to obtain what is termed herein the "crude product." Precipitated products were isolated by concentrating the above chloroform crude product solution to a total volume of ~4 mL and precipitation of the polymer in 30 mL of methanol. The resulting precipitate was washed with methanol and ether and then dried *in vacuo*. Unless otherwise specified, the isolated products were obtained using one precipitation/washing cyle.

Fractionation. The PHA formed in the medium with 4% PEG (cultivation condition B; see above) and isolated by one precipitation/washing cycle was dissolved in chloroform (0.1 g/mL). Acetone (10 volumes) was slowly added to the chloroform solution. The white cotton-like precipitate which resulted from acetone addition was isolated by filtration, giving the acetone insoluble (AIS) fraction, and the solvent was evaporated from the acetone−chloroform solution, which gave the acetone soluble (AS) fraction. Removal of residual solvents from the AS and AIS fractions was carried out in a vacuum desiccator (10 mmHg, 24 h), and the samples were then allowed to age for at least 1 week at ambient temperature prior to carrying out thermal analyses.

Polymer Characterization. A UNITY-500 NMR spectrometer was used for 1- and 2-D proton NMR experiments described below. Proton (^{1}H) NMR were recorded at 500 MHz. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters were as follows: 0.5% (w/v) polymer in chloroform-*d*, 298 K, 2.4 μs (14°) pulse width, 3 s acquisition time, and 6000 Hz spectral width. Carbon (^{13}C) NMR spectra were recorded at 101 MHz with the following parameters: 2.0% (w/v) polymer in chloroform-*d*; 298 K, 17.0 (90°) μs pulse width, 0.626 s acquisition time and 20 s pulse delay, 23 981 Hz spectral width, and 15 378−92 218 accumulations. The observed ^{13}C NMR chemical shifts (ppm) were referenced relative to chloroform-*d* at 76.91 ppm. For the COSY experiment (0.5% (w/v) polymer in chloroform-*d*) the data were collected in a 1024 × 256 data matrix and zero-filled to 1024 × 1024 using 8 scans/increment, a 4260 Hz sweep width, and a 1.1 s delay between transients. The data were processed using sinebell weighting.

The molecular weights of polyesters were determined by GPC. GPC studies were carried out using a Waters HPLC system with 500, 10^3, 10^4, and 10^5 Å Ultrastyragel columns placed in series. Chloroform was used as the eluent at a flow rate of 1.0 mL/min, sample concentrations were typically 10 mg/mL, and the injection volume was 100 μL. Polystyrene standards (Aldrich) with low polydispersities were used to generate a calibration curve from which product molecular weights were determined with no further corrections.

All thermal characterizations were carried out using a DuPont 2910 differential scanning calorimeter equipped with a TA 2000 data station, using between 5.0 and 6.0 mg of sample sealed in aluminum pans and a dry nitrogen purge. The polymer samples were heated at a rate of 10 °C/min from room temperature to 200 °C, rapidly quenched by liquid nitrogen from the melt, and then analyzed during second heating scans from −80 to 200 °C. Data reported for the melting temperature(s), T_m, and enthalpy of fusion(s), ΔH_f, were taken from the first heating scan. The reported glass transition temperature (T_g) values were the midpoint values measured during the second heating scans.

Results and Discussion

Effect of PEG-200 on PHA Repeat Unit Composition, Yield, and Molecular Weight. Control of the composition for copolyesters of 3HB and 4HB is normally achieved by variation in the carbon sources used or by alteration of other physiological parameters such

Table 1. Effects of PEG-200 on the Production and Compositions of Microbial Polyesters Formed by *A. eutrophus* using 4-Hydroxybutyrate as the Carbon Source

polym,[a] % PEG	culture condition	cell yield,[d] g/L	polym content of cells,[e] %	PHA yield,[f] g/L	repeat units found in PHA, mol %				M_n,[g] g/mol × 10^{-3}	M_w/M_n[g]
					3HB	4HB	3HV	EG		
0[b]	A[h]	3.7	21	0.76	34	66	0	0	222.1	2.76
1[b]	A	3.5	16	0.56	20	79	1.1	0	178.6	1.89
2[b]	A	3.1	14	0.45	11	86	2.8	0.28	153.0	2.05
4[b]	A	2.6	14	0.37	30	64	5.0	0.93	112.2	2.51
0[b]	B[i]	3.9	27	1.1	70	30	0	0	198.6	2.87
4[b]	B[i]	3.7	26	0.97	41	53	5.4	1.1	77.2	3.95
4-AS[c]	B				13	84	2.1	1.6	37.4	2.52
4-AIS[c]	B				95	3	2.0	0.1	130.0	3.42

[a] The % PEG added to the cultivation medium during the second or polymer producing stage. [b] Nonfractionated samples obtained from one precipitation/washing cycle. [c] AS and AIS are the acetone soluble (57% (w/w)) and insoluble (43%) fractions of the 4% PEG product obtained using cultivation condition B (see *i* below). [d] The quantity of harvested cells after they were washed with Nanopure water and lyophilized (see Experimental Section). [e] Expressed as the percent of the cellular dry weight which contains PHA. These values were obtained gravimetrically from the isolated product from chloroform extraction and one precipitation/washing cycle (see Experimental Section). [f] PHA yield = (cell yield) (fraction of the cellular dry weight which is PHA). [g] Determined by GPC (see Experimental Section). [h] Cultivation condition A used 100 mL of media in a 500 baffled shake flask (see Experimental Section). [i] Cultivation condition B used 500 mL of media in a 2.8 L shake flask (see Experimental Section).

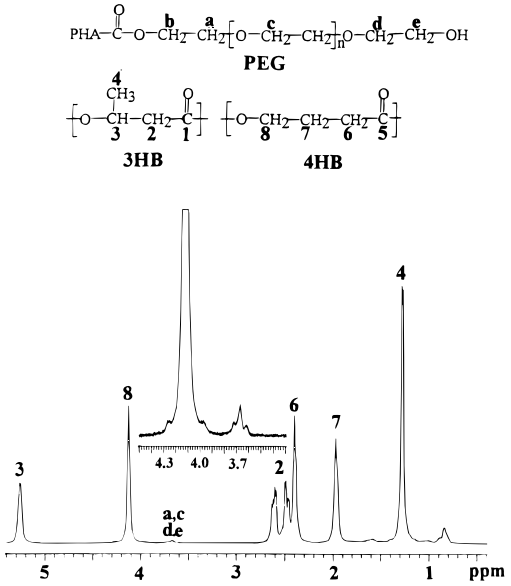

Figure 1. 500 MHz ^{1}H NMR spectrum of the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B).

as the incubation time and nitrogen concentration.[2,31] In this study, PEG-200 was added to *A. eutrophus* cultivations in concentrations up to 4% (w/v) during the second or polymer producing stage of the fermentation (see Experimental Section) where 4-hydroxybutyrate served as the carbon source. The mole fractions of repeat units for PHAs isolated by one precipitation per washing cycle (see Experimental Section) were analyzed by ^{1}H NMR spectral integration of well-resolved signal regions (see Figure 1) as has been previously described.[31,33] Table 1 shows that, upon the addition of PEG-200 for culture conditions A, dramatic shifts in the repeat unit composition were achieved. Specifically, the mole percent of 4HB in the product changed from 66 to 86% by the addition of 0 and 2% PEG-200, respectively. Upon further addition of PEG-200 up to 4%, the mole percent of 4HB decreased. Furthermore, the addition of PEG-200 resulted in products containing low-level incorporations of 3HV repeat units (see Table 1). Thus, small quantities of PEG-200 added to fermentation media caused important product compositional changes. This experiment was repeated, and the identical trends were observed. It should be noted that a change in the cultivation method from A to B (see Experimental Section) resulted in products of different repeat unit composition (see Table 1). This is not surprising since the cultivation volume and flask size are important variables that ultimately alter culture physiological conditions such as oxygen transfer rate and can result in the formation of different products.[43] The formation in PEG amended *A. eutrophus* cultivations of odd chain 3HV repeat units from even chain 4-hydroxybutyric acid is interesting since it indicates that PEG functions by an at present unknown mechanism to shift the metabolism from even to odd chain products.

Table 1 also provides results on the effects of PEG-200 on volumetric yield and product molecular weight for the series of fermentations carried out under culture conditions A. The volumetric yield of the PHAs continued to decrease with increased PEG media concentration so that for 2 and 4% PEG-200 addition the yields were approximately 59 and 48%, respectively, of that for PEG deficient media (see Table 1). The M_n and M_w/M_n values measured by GPC of the products formed from cultivations with 0, 1, and 2% PEG are shown in Table 1. The GPC traces of these products were unimodal. M_n was found to decrease with increasing PEG-200 concentration in the media. Since we have not taken into account changes in polymer hydrodynamic volume as a function of chain composition, the observed molecular weight decrease shows a trend but does not provide absolute values. However, it is important to note that, in a previous study by us, increasing the media PEG-200 concentration from 0 to 1% resulted in a large decrease in the molecular weight of P3HB formed by *A. eutrophus* from fructose.[45] Interestingly, the GPC traces for the products formed in fermentations amended with 4% PEG-200 (culture conditions A and B, see Experimental Section and Table 1) were very complex and are discussed in detail below.

The ^{1}H NMR spectrum of the PHA isolated by one precipitation/washing cycle for a cultivation containing 4% PEG-200 (culture condition B, see Table 1) is shown in Figure 1. Interestingly, weak ^{1}H NMR signals at ~3.7 ppm were observed that correspond to protons of ethylene glycol (EG) repeat units. P(3HB-*co*-4HB) formed in the absence of PEG does not show ^{1}H NMR signals in the 3.6−3.8 ppm spectral region. A COSY spectrum of this product was recorded, and the specific spectral regions of interest are shown in Figure 2. Three signals at 4.25, 4.35, and 4.46 ppm were observed that have correlations with signals at 3.70, 3.73, and 3.77 ppm, respectively. The signal at 4.25 ppm also has

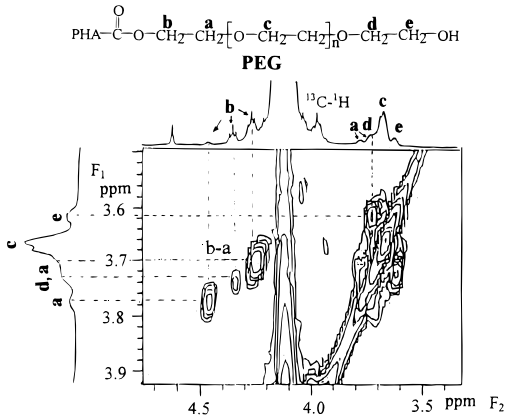

Figure 2. Expansion of the two-dimensional homonuclear (^{1}H) correlated (COSY) spectrum of the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B).

a contribution from a satellite peak of protons *8* (4.1 ppm) due to ^{13}C−^{1}H coupling. On the basis of chemical shift parameters documented for model compounds,[46a,b] it is expected that esterification of a terminal PEG−CH_2−OH will lead to a downfield shift from ∼3.7 to ∼4.25 ppm. Considering the above, the signals in the 3.68−3.80 and 4.20−4.50 ppm regions were assigned to protons *a* and *b*, respectively, of esterified PEG chain segments. Correlation of the signals with peaks at 3.62 and 3.73 ppm suggest that they are due to protons *e* and *d* of terminal free hydroxyl EG units (see Figure 2). Assuming that the contribution of the overlapping signals in the 3.6−3.8 ppm region can be estimated by Bernoullian curve fitting, the area under the peaks was measured by cutting and weighing. The integration results showed that the ratio of protons $a + d$ to *c* to 2*e* was 3:6:2. Using the ratio *c* to $a + d$ and *c* to 2*e* gives values of *n* (internal EG units) of 2 and 3, respectively. Thus, the average chain length of PEG segments is between 4 and 5 which corresponds to molecular weights of ∼180 and 220 g/mol, respectively. The above results are consistent with the formation of PHA chains that are covalently linked at the carboxylate chain terminus to PEG chain segments. In other words, we believe that PHA−PEG diblock copolymers were formed (see Figures 1 and 2). Furthermore, the average PEG chain length in the product is almost identical to that which was provided to the cultivation media. Deviations from 1 of the peak area ratio $a + d$ to 2*e* (3:2) as well as inherent uncertainty in peak area approximations and the inability to estimate the peak area of protons *b* due to overlap with a ^{13}C−^{1}H satellite peak makes it unclear at present that PEG is found exclusively at chain terminal positions. Using spectrometer integration of the signals at 1.25 ppm (protons *4*), 1.95 ppm (protons *7*), 0.82 ppm (methyl protons of 3HV), and 3.6−3.8 ppm (protons *a*, *c*, *d*, and *e*), it is estimated that the isolated product contains 1.1 mol % and, therefore, 0.2% (w/w) of EG repeat units.

To provide further evidence that ^{1}H NMR signals observed in the 3.6−3.8 ppm region for one time precipitated products are not due in part to residual PEG-200, the following experiments were performed. P(3HB-*co*-30% 4HB) (produced by a cultivation of *A. eutrophus* with no added PEG-200, see Table 1) and PEG-200 (286 and 218 mg, respectively) were dissolved in chloroform and cast to form a film. This film which contained 43% by weight PEG-200 which exceeds by a factor of ∼2 times the quantity of PEG-200 found in the

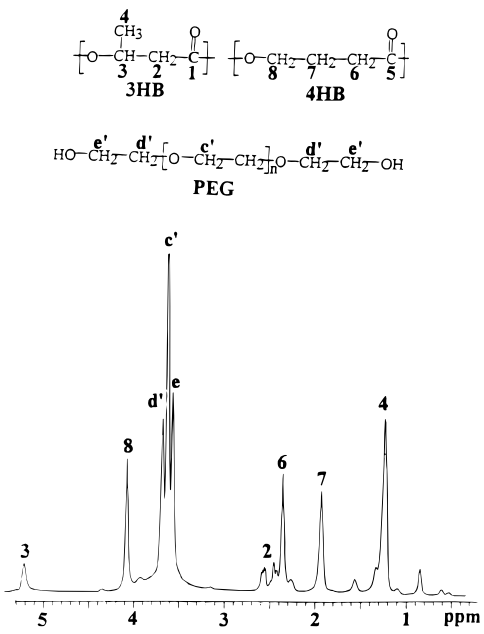

Figure 3. 500 MHz ^{1}H NMR spectrum of the chloroform extracted crude product from a cultivation with media containing 4% PEG-200 (culture conditions B).

corresponding crude product (nonprecipitated solution extracted material; see discussion below) was then purified by one precipitation/washing cycle using identical conditions as were used for isolated products. The resulting isolate contained 0 mol % PEG from investigations by ^{1}H NMR. Also, PEG-200 (3 parts) was mixed with a PHA−PEG product (7 parts) that was obtained after three precipitation/washing cycles from 4% PEG amended cultivations. Once again, after only one precipitation/washing cycle, the relative signal intensities of the 3.6−3.8 ppm signal region to PHA protons was identical to that of the PHA−PEG product prior to mixing with PEG-200. Moreover, repeated precipitation/washing (up to three times) of one time precipitated samples did not result in a change in the EG mole percent. Therefore, it is concluded that non-covalently linked PEG-200 is indeed removed efficiently from the isolated products by one precipitation/washing cycle. This supports that PEG found in isolated products is covalently linked to PHA chains.

Studies on the Crude Product. The above results demonstrate that PEG-200 added to *A. eutrophus* cultivation media does indeed interact with the cellular PHA biosynthetic system. We then considered to what extent PEG-200 was taken into intact cells. This was studied by chloroform extraction of the lyophilized cells obtained from a 4% PEG-200 amended media fermentation (culture condition B; see Table 1), removal of insoluble cellular material, and evaporation of the solvent (see Experimental Section). The resulting crude product fraction was analyzed by both ^{1}H NMR (Figure 3) and GPC (Figure 4). Using ^{1}H NMR spectrometer integration, as described above, the mole percent of PEG, 3HB, 4HB, and 3HV repeat units are 20, 34, 42, and 3, respectively. This corresponds to a weight ratio of PEG to PHA in harvested cells of 1−1.7 (assuming the contribution of PEG which is in the form of PHA−PEG to be relatively small; see Figure 1). From the above and values of the dry cell and PHA−PEG yields (3.7 and 0.97 g/L, respectively; see Table 1), it is estimated that ∼9.5% of the cellular dry weight contains PEG. Spectrometer integration of terminal PEG−CH_2−

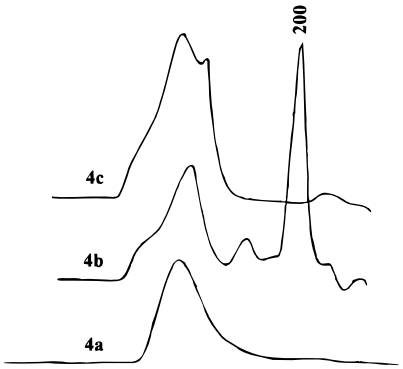

Figure 4. GPC traces of (a) the crude product from a cultivation with media containing 0% PEG-200 (culture conditions B), (b) the sample described in the legend of Figure 3, and (c) the sample described in the legend of Figure 1.

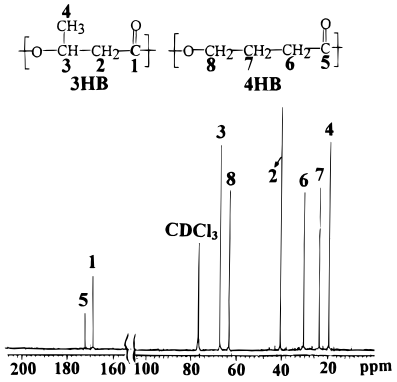

Figure 5. 125 MHz ^{13}C NMR spectrum of the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B).

OH protons *e′* at 3.58 ppm relative to internal HO(−OCH_2CH_2−)$_n$−OH *c′* protons at 3.65 ppm showed that the M_n of PEG accumulated in cells was 194 g/mol. Thus, it appears that when *A. eutrophus* is placed in 4% PEG-200 amended media under polymer producing conditions, the cells respond to the PEG external stimulus by accumulating extraordinarily large quantities of oligomeric PEG that has an M_n closely resembling that of the PEG added to cultivation media.

The GPC trace of the extracted crude material from cultivations (culture condition B; see Table 1) containing 4% PEG was quite complex, indicating that it is a mixture of products having very different molecular weight averages (see Figure 4, trace 4b). However, the GPC trace of the crude polymer product obtained from cultivation media without PEG-200 (see Figure 4, trace 4a) shows only a unimodal peak. Also, it is interesting to note that the GPC of the crude product has a component peak with an elution volume which corresponds exactly with that of PEG-200. This is further evidence that PEG-200 does indeed accumulate in cells and that this occurs without notable cellular selectivity as a function of PEG chain length. On the basis of a standard curve constructed of GPC peak area versus PEG-200 concentration (not shown), it is estimated that ∼10% of the cellular dry weight contains oligomers similar in molecular weight to PEG-200. This result is in good agreement with that given above (9.5%) determined by ^1H NMR spectrometer integration. Consistent with the studies above where PEG-200 was mixed with PHAs and removed by one precipitation/washing cycle, the GPC trace of the one time precipitated product shows no trace of residual PEG-200 but still shows multiple component peaks. Since the question of PEG chain length selectivity by *A. eutrophus* is important, work is currently in progress in our laboratory to gain further information by using variable PEG chain lengths in culture media and determining the distribution of intracellular PEG oligomers by gas chromatographic methods.[47]

Repeat Unit Sequence Distribution. Effects of PEG-200. PHAs isolated from *A. eutrophus* cultivations where 4-hydroxybutyrate served as a carbon source and PEG-200 was not added to the media formulations were reported in the literature as having sequence distributions of 3HB and 4HB repeat units that are approximately random.[31] For investigation of the effects of PEG-200 on the repeat unit sequence distribution, the ^{13}C NMR spectra for the one time precipitated/washed products obtained from fermentations with 0 and 4% PEG-200 (culture condition B) were recorded.

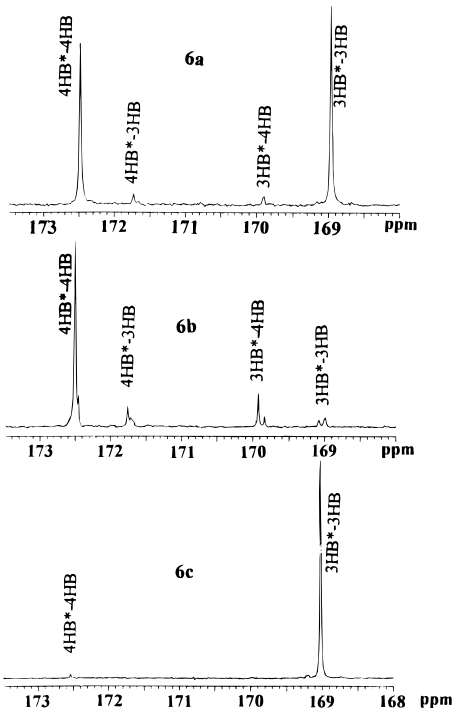

Figure 6. Expansions of the carbonyl region from 101 MHz ^{13}C NMR spectra of (a) the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B), (b) the acetone soluble (AS) fraction of the product described in a, and (c) the acetone insoluble (AIS) fraction of the product described in a.

The ^{13}C NMR spectrum for the latter product as well as an expansion of the carbonyl region is shown in Figures 5 and 6a, respectively. Assignment of signals observed, including those in the carbonyl region which are sensitive to effects of repeat unit sequence distribution, are based on previous work.[31,33,34]

For simplification of the repeat unit sequence analysis below, the small contributions from 3HV and EG repeat units will be neglected so that the products will be assumed to consist of only 3HB and 4HB repeat units. The relative mole fractions of 3HB*−3HB (3*3), 3HB*−4HB (3*4), 4HB*−3HB (4*3), and 4HB*−4HB (4*4) diads (see Figure 6) were determined by spectrometer integration and are given in Table 2. Experimental values are compared to those calculated assuming a Bernoullian or random statistical process for microbial catalyzed copolymerization using the following relationships (eqs 1−3) where F_3 is the mole fraction of 3HB units in the polymer:[33,48,49]

Table 2. Experimental[a] and Calculated[b] Comonomer Diad Fractions for PHAs and Product Fractions Formed in Cultivations with and without PEG-200

	diad sequence			
	3HB−3HB	3HB−4HB	4HB−3HB	4HB−4HB
polym,[c] % PEG	exptl[a] (calcd[b])	exptl[a] (calcd[b])	exptl[a] (calcd[b])	exptl[a] (calcd[b])
O[e]	0.58 (0.49)	0.13 (0.21)	0.11 (0.21)	0.18 (0.10)
4[d,e]	0.48 (0.32)	0.03 (0.25)	0.03 (0.25)	0.46 (0.18)
4-AS[f]	0.07 (0.03)	0.11 (0.15)	0.11 (0.15)	0.71 (0.67)
4-AIS[f]	0.98 (0.85)	0 (0.07)	0 (0.07)	0.02 (0.01)

[a] Determined by measuring the relative peak areas for the carbonyl carbon ^{13}C NMR signals assigned (see Figure 6) to the four diad sequences. [b] Calculated values from eqs 1−3, assuming a Bernoullian or random statistical process and that the contribution of 3HV and EG repeat units can be neglected. [c] The % PEG added to the cultivation medium during the second or polymer producing stage. [d] The nonfractionated sample obtained from one precipitation/washing cycle. [e] The cultivation was carried out using culture condition B (500 mL of media in a 2.8 L shake flask; see Experimental Section). [f] AS and AIS are the acetone soluble (57% (w/w)) and insoluble (43%) fractions of the 4% PEG product (see *d* above).

$$[3^*] = F_3^2 \tag{1}$$

$$[3^*4] = [4^*3] = F_3(1 - F_3) \tag{2}$$

$$[4^*4] = (1 - F_3)^2 \tag{3}$$

By comparison of experimental and calculated results given in Table 2 for the PHA produced with 0% PEG, it appears that this product approximates a statistically random copolymer, as was previously indicated in the literature.[28,31,33] However, it should be noted that there is a small but significant deviation between calculated and experimental values. In contrast, the addition of 4% PEG to cultivations resulted in a product that has predominantly 3HB*−3HB and 4HB*−4HB diads (Table 2 and Figure 6a). The DSC thermograms of this product during a first heating scan show two distinct T_m values at 55 and 170 °C (Figure 7a, Table 3) which closely approximate reported T_m values for P3HB and P4HB (54 and 177 °C, respectively).[30] The DSC thermogram of this product recorded during a second heating scan after rapidly quenching from the melt (see Experimental Section) showed T_g values at −45 and −29 °C (Figure 8a, Table 3). The T_g at −45 °C closely approximates that reported for P4HB (−50 °C), while the T_g at −29 °C is intermediate to those reported for P3HB (∼4 °C [31]) and P4HB. The observed T_g at −29 °C is interesting and may result from the formation of a small product fraction that consists of random 3HB/4HB copolyester chains having the corresponding T_g value. Thus, the results of DSC and ^{13}C NMR studies both support that the product formed in the 4% PEG-200 amended medium may be (1) a mixture of chains that approximate P3HB and P4HB homopolymers, (2) a copolyester with long P3HB and P4HB chain segments, or (3) some combination of the first two possibilities.

Studies on Fractionation of the Product from 4% PEG-200 Cultivations. If the product obtained is a mixture of polyesters as opposed to a block copolymer, then it should be possible to fractionate the product into component polyesters based on differential solubility characteristics. Fractionation based on the solubility of the product in acetone was carried out (see Experimental Section). This resulted in acetone soluble (AS, 57% w/w) and insoluble (AIS, 43% w/w) fractions with

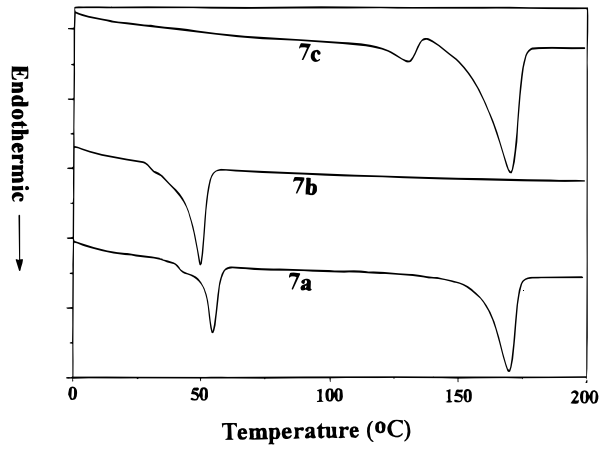

Figure 7. DSC thermograms (first heating scans) of (a) the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B), (b) the acetone soluble (AS) fraction of the product described in a, and (c) the acetone insoluble (AIS) fraction of the product described in a.

Table 3. Thermal Analysis Obtained by DSC[a] Measurements

polym,[b] % PEG (w/v)	T_g,[e] °C	T_m,[f] °C	ΔH,[g] cal/g
4[c,j]	−45	55	5.0
	−29	170	11.6
4-AS[d]	−42	50	8.8
	−15		
4-AIS[d]	2.8	172	19.0

[a] A scanning rate of 10 °C/min was used. [b] The % PEG added to the cultivation medium during the second or polymer producing stage. [c] The nonfractionated sample obtained from one precipitation/washing cycle. [d] AS and AIS are the acetone soluble (57% (w/w)) and insoluble (43%) fractions of the sample presented as the second table entry. [e] Glass transition temperatures taken as the midpoint of the heat capacity change and measured during the second heating scan after rapidly quenching by liquid nitrogen at −70 °C from the melt. [f] Peak melting temperatures for each endothermic melting transition determined during the first heating scan. [g] The heat of fusion value measured for each melting endothermic transition. [h] The cultivation was carried out using 500 mL of media in a 2.8 L shake flask (see Experimental Section).

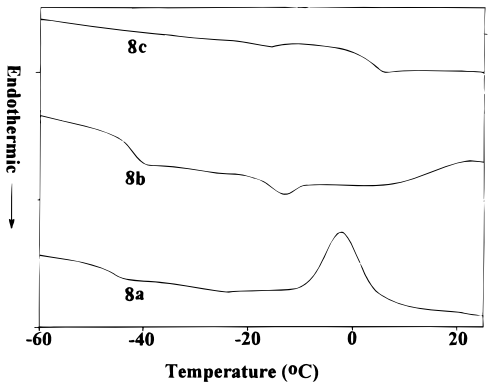

Figure 8. DSC thermograms (second heating scans) of (a) the purified product from a cultivation with media containing 4% PEG-200 (culture conditions B), (b) the acetone soluble (AS) fraction of the product described in a, (c) the acetone insoluble (AIS) fraction of the product described in a.

M_n (M_w/M_n) values of 37 400 (2.53) and 130 000 (3.42), respectively (see Table 1). Analysis of the repeat unit composition of these fractions by ^1H NMR spectral integration (see Table 1) showed that the mole fractions of 3HB, 4HB, 3HV, and EG repeat units are 13, 84, 2, 1.6 and 95, 3, 2, 0.1, respectively (see Table 1). Thus,

the addition of PEG-200 to cultivation media results in the formation of a new product that is a blend of polyesters. The formation of a mixture as opposed to random 3HB/4HB copolyesters is unusual based on previous studies[28,31,34] but was reported once before from cultivations for extended times of *A. eutrophus* on a mixture of γ-butyrolactone and butyric acid.[50] Also, it is interesting to note that PEG chain segments are found primarily in the AS high 4HB fraction (see Table 1). This is preliminary evidence that, for *A. eutrophus*, linkages between PEG and PHA segments may occur primarily between 4HB and EG repeat units. Using the model where it is assumed that PEG segments are at all carboxyl terminal positions of PHA chains in the AS fraction, the M_n calculated molecular weight based on ^{1}H NMR spectral integration is 24 000 g/mol, whereas the experimentally determined value from GPC is 37 400 g/mol. Considering that molecular weight measurements by GPC were determined relative to polystyrene standards and inherent error associated with both GPC and ^{1}H NMR derived molecular weight values, it is not possible at this stage to use this information as support for the above hypothesis. However, it is clear that these results are not inconsistent with this possibility. Additional experimentation is currently underway to further test this hypothesis.

Further information as to the repeat unit sequence distribution and complexity of the product formed in 4% PEG amended media was gained by ^{13}C NMR and DSC measurements of the AS and AIS fractions. Expansions of the ^{13}C NMR carbonyl spectral regions for these fractions are shown in Figure 6b,c, respectively. DSC thermograms of the first and second heating scans are shown in Figures 7 and 8, respectively. If the T_g, T_m, and ΔH_f values for solution precipitated P3HB are taken as 4 °C, 20.8 cal/g, and 177 °C based on a previous report,[31] comparison of these data to those obtained for the AIS fraction (see Table 3) indicates that this fraction contains primarily P3HB homopolymer as opposed to a random copolyester such as P(3HB-*co*-6 mol % 4HB) that was reported to have T_m and ΔH_f values of 162 °C and 13.5 cal/g, respectively.[31] This is further supported by the ^{13}C NMR spectrum of the AIS fraction which shows only 3HB*−3HB diads (see Figure 6c). The diad sequence distribution of the AS fraction determined experimentally (see Figure 6b) and calculated using eqs 1−3 above suggests that the product formed approximates that of a random copolyester (see Table 2). Further study of this fraction by DSC indicates product heterogeneity. Specifically, the AS fraction has multiple T_g (−15, −42 °C) transitions and a broad melting region (see Table 3, Figures 7b and 8b). It is once again of interest to compare the thermal transitions of this product fraction to those previously reported for 3HB/4HB random copolyesters.[31] Specifically, the T_g values of the AS fraction (−42, −15 °C) closely match those for P(3HB-*co*-90% 4HB) and P(3HB-*co*-28% 4HB) random copolyesters (−44 and −15 °C, respectively).[31] From this analysis and with the assumption that the components are immiscible, we estimate that the AS fraction is a mixture of random copolyesters with relatively high and low 4HB contents. Thus, it appears that the unfractionated product from media containing 4% PEG is indeed complex as was originally indicated by the GPC trace (see Figure 4c, above) and is composed of at least three different component polymers of different repeat unit composition.

Other Considerations. Microbial polyesters are formed in intracellular inclusion bodies or granules[51] that possess a membrane-like coating. The coating is composed of lipid and protein that has been reported for granules of *Bacillus megaterium* to represent 0.5−2%, respectively, of the granule weight.[52] It has been shown that the core of nascent granules are amorphous.[53,54] The membrane coat surrounding PHA granules has been reported to be approximately 2 nm thick[51,53,55] and may consist of a lipid monolayer.[55] A hypothesis that is currently being investigated is that PEG taken into cells develops specific interactions with granule outer membrane proteins and likely accumulates within the amorphous core of granules. Studies are currently in progress using isolated granules as well as whole cells to identify whether PEG accumulates at specific locations within cells.[56] Our current view is that the results of introducing PEG in *A. eutrophus* cultivation media are not unlike what occurs when an effective pharmaceutical agent is used in that both bioactive agents target certain biochemical systems and cause specific changes in metabolism.

Summary of Results

The introduction of PEG-200 as a bioactive agent was explored for cultivations of *A. eutrophus*. The results herein indicate that PEG-200 interacts with enzyme systems involved in PHA biosynthesis to cause dramatic product structural modulation. Specifically, PEG-200 addition to culture media resulted in the following: (1) changes in the mole percent of 3HB, 3HV, and 4HB repeat units, (2) formation of complex product mixtures composed of an AS fraction and an AIS fraction with high 4HB and 3HB contents, respectively, and (3) a method to prepare PHA−PEG diblock copolymers where the carboxylate terminus of PHA chains are covalently linked by an ester bond to PEG chain segments. It is noteworthy to mention that, to our knowledge, the PHA−PEG diblock copolymer reported herein is the first example of the *in vivo* formation of a natural−synthetic block copolymer.

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References and Notes

(1) Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller, R. C. in *Advances in Biochemical Engineering/Biotechnology*; Ghose, T. K., Fiechter, A., Eds.; Springer: Berlin, 1990; Vol. 41, p 77.
(2) Doi, Y. *Microbial Polyesters*; VCH: New York, 1990.
(3) Steinbüchel, A. In *Biomaterials: Novel Materials from Biological Sources*; Byrom, D., Ed.; Stockton Press: New York, 1991; Chapter 3, p 123.
(4) Gross, R. A. Bacterial Polyesters: Structural Variability in Microbial Synthesis. In *Biomedical Polymers: Designed-to-Degrade Systems*; Shalaby, S. W., Ed.; Hanser: New York, 1994; pp 173−188.
(5) Nishida, H.; Tokiwa, Y. *J. Environ. Polym. Deg.* **1993,** *1* (3), 227.
(6) Mergaert, J.; Webb, A.; Anderson, C.; Wouters, A.; Swings, J. *Appl. Environ. Microbiol.* **1993,** *59*, 3233.
(7) Mergaert J.; Anderson, C.; Wouters, A.; Swings, J. *J. Environ. Polym. Deg.* **1994,** 2 (3), 177.
(8) Mayer, J. M.; Greenberger, M.; Kaplan, D. L. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1990,** *31* (3), 858.
(9) Budwill, K.; Fedorak, P. M.; Page, W. *J. Appl. Environ. Microbiol.* **1992,** *58* (4), 1398.
(10) Winton, J. M. *Chem. Week*, **1985** (Aug 28), 55.
(11) Uttley, N. L. *Appl. Biotechnol. Proc. BIOTECH'86* **1986,** *1*, 171.

(12) Lemoigne, M. *Ann. Inst. Pasteur (Paris)* **1925,** *39*, 144.
(13) Lemoigne, M. *Bull. Soc. Chim. Biol.* **1926,** *8*, 770.
(14) Lemoigne, M. *Ann. Inst. Pasteur (Paris),* **1927,** *41*, 148.
(15) Azuma, Y., Yoshie, N.; Sakurai, M.; Inoue, Y.; Chujo, R. *Polymer,* **1992,** *33* (22), 4763.
(16) Howells, E. M. *Chem. Ind.* **1982,** 508.
(17) Holmes, P. A. *Phys. Technol.* **1986,** *16*, 32.
(18) King, P. P. *J. Chem. Technol. Biotechnol.* **1982,** *32*, 2.
(19) Brandl, H.; Knee, E. J., Jr.; Fuller, R. C.; Gross, R. A.; Lenz, R. W. *Int. J. Biol. Macromol.* **1989,** *11*, 49.
(20) Shiotani, T.; Kobayashi, G. Japanese Pat. Appl. 93049, 1993.
(21) Shimamura, E.; Kasuya, K.-I.; Kobayashi, G.; Shiotani, T.; Shima, Y.; Doi, Y. *Macromolecules* **1994,** *27*, 878.
(22) Steinbüchel, A.; Debzi, El-M.; Marchessault, R. H.; Timm, A. *Appl. Microbiol. Biotechnol.* **1993,** *39*, 443.
(23) Mayer, J. M.; Kaplan, D. L. *Trends Polym. Sci.* **1994,** *2* (7), 227.
(24) Kim, Y. B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1991,** *24*, 5256.
(25) Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Makromol. Chem.* **1990,** *191*, 1957.
(26) Kim, O.; Gross, R. A. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.*) **1994, 35** (2), 627.
(27) Kim, O.; Gross, R. A.; Rutherford, D. R. *Can. J. Microbiol.*, in press.
(28) Kunioka, M.; Nakamura, Y.; Doi, Y. *Polym. Commun.* **1988,** *29*, 174.
(29) Kunioka, M.; Kawaguchi, Y.; Doi, Y. *Appl. Microbiol. Biotechnol.* **1989,** *30*, 569.
(30) Doi, Y.; Segawa, A.; Kunioka, M. *Int. J. Biol. Macromol.* **1990,** *12*, 106.
(31) Nakamura, S.; Doi, Y.; Scandola, M. *Macromolecules* **1992,** *25*, 4237.
(32) Kimura, H.; Yoshida, Y.; Doi, Y. *Biotechnol. Lett.* **1992,** *14* (6), 445.
(33) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1988,** 21, 2722.
(34) Doi, Y.; Segawa, A.; Kunioka, M. *Polym. Commun.* **1989,** *30*, 169.
(35) Steinbuchel, A.; Valentin, H. E.; Schonebaum, A. *J. Environ. Polym. Deg.* **1994,** *2* (2), 67.
(36) Harris, J. M. Introduction to Biotechnical and Biomedical Applications of Poly(ethylene glycol). In *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*; Harris, J. M., Ed; Plenum Press: New York, 1992; pp 1−14.
(37) Inada, Y.; Matsushima, A.; Kodera, Y.; Nishimura, H. *J. Bioact. Compat. Polym.* **1990,** *5*, 343.
(38) Albertsson, P.-Å. *Partition of Cell Particles and Macromolecules*, 3rd ed.; Wiley: New York, 1986.
(39) Yamazaki, M.; Ito, T. *Biochemistry* **1990,** *29*, 1309.
(40) Boni, L. T.; Hah, J. S.; Hui, S. W.; Mukherjee, P.; Ho, J. T.; Jung, C. Y. *Biochem. Biophys. Acta* **1984,** *775*, 409.
(41) Ingram, L. O.; Buttke, T. M. Effects of Alcohols on Microorganisms. In *Advances in Microbiology and Physiology*; Rose, A. H., Tempest, D. W., Eds.; Academic Press: New York, 1984; Vol. 25, pp 253−300.
(42) Santos-Díaz, M. del-S.; Ochoa-Alejo, N. *Plant Cell Tissue Organ Cult.* **1994, 37**, 1.
(43) Parton, C.; Willis, P. in *Fermentation, A Practical Approach*; McNeil, B., Harvey, L. M., Eds.; IRL Press: Oxford, U.K., 1990; Chapter 3.
(44) Irvine, T. S. In *Fermentation, A Practical Approach*; McNeil, B., Harvey, L. M., Eds.; IRL Press: Oxford, U.K., 1990; Chapter 2.
(45) Shi, F. Y.; Rutherford, D. R.; Gross, R. A. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1995,** *36*, 430.
(46) (a) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Indentification of Organic Compounds*, 4th ed.; (b) Bovey, F. A. *High Resolution NMR of Macromolecules*; Academic Press: New York, 1972.
(47) Fletcher, J. P., Persinger, H. E. *J. Polym. Sci.. Part A-1* **1968,** *6*, 1025.
(48) Randall, J. C. *Polymer Sequence Determination*; Academic Press: New York, 1977; Chapter 4.
(49) Bluhm, T. L.; Hamer, G. K.; Marchessault, R. H.; Fyfe, C. A.; Veregin, R. P. *Macromolecules* **1986,** *19*, 2871.
(50) Doi, Y. *Microbial Polyesters*; VCH: New York, 1990; Chapter 5.
(51) Dunlop, W. F.; Robards, A. W. *J. Bacteriol.* **1973,** *114*, 1271.
(52) Griebel, R.; Smith, Z.; Merrick, J. M. *Biochemistry* **1968,** *7*, 3676.
(53) Koning, G. J. M.; Lemstra, P. *J. Polymer* **1992,** *33* (15), 3292.
(54) Lauzier, C.; Marchessault, R. H.; Smith, P.; Chanzy, H. *J. Polymer* **1992,** *33* (4), 823.
(55) Preusting, H.; Kingma, J.; Witholt, B. *Enzyme Microb. Technol.* **1991,** *13*, 770.
(56) Studies with whole cells using deuterated PEG and neutron scattering techniques are being carried out in collaboration with Bill Orts at NIST, Gaithersburg, MD. Work has also been initiated using cell free models in collaboration with Ken Gruys at Monsanto Agricultural Missouri Co., St. Louis, MO.

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