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Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture

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In the present work, attention was devoted to understand how different carbon substrates and their concentration can influence the production of PHA by polyphosphate-accumulating bacteria. Acetate, propionate, and butyrate were tested independently. The composition of the polymers formed was found to vary with the substrate used. Acetate leads to the production of a copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV) with the HB units being dominant. With propionate, HV units are mainly produced and only a small amount of HB is synthesized. When butyrate is used, the amount of polymer formed is much lower with the HB units being produced to a higher extent. The yield of polymer produced per carbon consumed ($Y_{P/S}$) was found to diminish from acetate (0.97) to propionate (0.61) to butyrate (0.21). Using a mixture of acetate, propionate, and butyrate and increasing the carbon concentration, although maintaining the relative concentration of each substrate, propionate is primarily consumed and consequently, PHA synthesized was enriched in HV units. The polymers obtained in all experiments were copolymers with the average molecular weight of the most representative fraction higher when hydroxybutyrate units were present in considerable amounts. All the polymers synthesized were found to be quite homogeneous and their average molecular weight is of the same order of magnitude as the ones commercially available. © *1998 Elsevier Science Inc.*

Keywords: Polyhydroxyalkanoates; polymer characterization; biological phosphorus removal; activated sludge; carbon source

Introduction

Contamination of wastewaters with nitrate and phosphate from industrial and agricultural activities leads to eutrophication of water streams and lakes. It was found that wastewaters submitted to anaerobic/aerobic cycles mainly for nitrogen removal were also able to remove phosphorus in excess when compared with the microorganisms metabolic requirements. These microorganisms, known as polyphosphate bacteria, are capable of storing phosphate intracellarly as polyphosphate up to 10% of their dry weight.[1] By removing bacteria, phosphate can be removed at the same time. In recent years, this process has gained greater importance when compared with the chemical removal of phosphate. Some advantages of the biological process are low sludge production and the fertilizer value of the sludge. Several industrial treatment plants are working worldwide using this biological process.[2]

The metabolism of polyphosphate bacteria is not yet completely elucidated (*Figure 1*). It is known that these bacteria under anaerobic conditions can uptake short chain fatty acids, thereby producing polyhydroxyalkanoates (PHA) with simultaneous phosphorus release to the external medium.[3] Energy for this process comes from the breakdown of intracellular polyphosphate; the reducing equivalents needed are originated from glycogen consumption[4] and substrate degradation in the tricarboxylic acid cycle.[5] During the aerobic stage in the process, PHA previously accumulated in anaerobiosis is metabolized for anabolic precursors and microorganism growth. Part of the energy

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Received 14 April 1997; revised 17 October 1997; accepted 30 October 1997

655 Avenue of the Americas, New York, NY 10010
0141-0229/98/$19.00
PII S0141-0229(97)00243-3

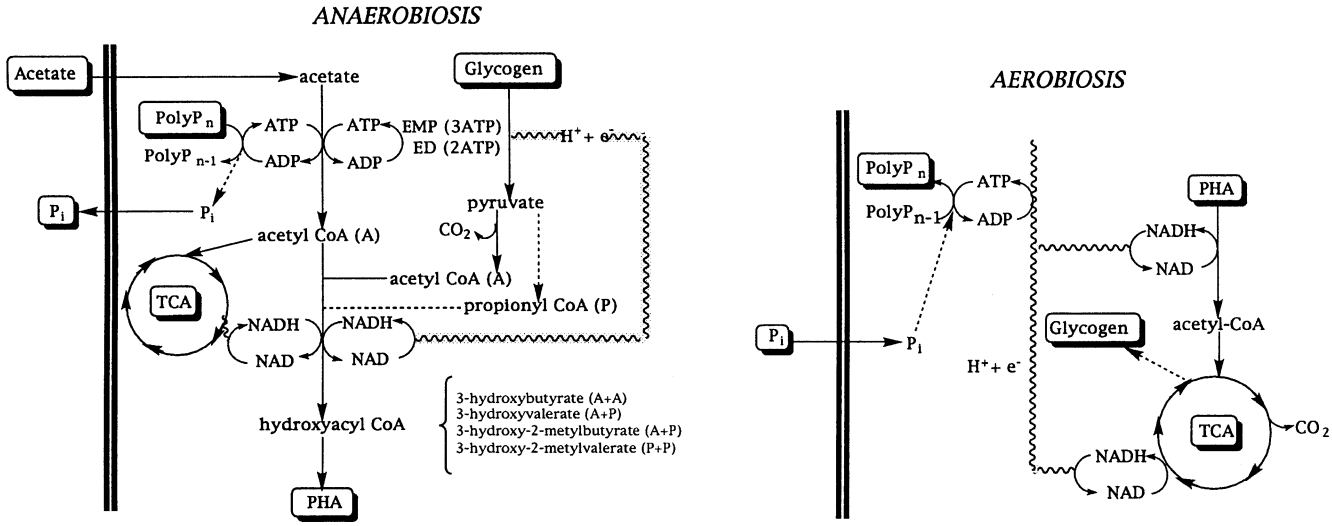

Figure 1 Carbon (acetate) and phosphorus metabolism for polyphosphate bacteria during anaerobiosis and aerobiosis (according to Pereira *et al.*[5])

produced is used for synthesis of polyphosphate with phosphate uptake from the external medium. Also some of the precursors are used for glycogen formation. It has been shown that these microorganisms have three different types of intracellular reserve products: polyphosphate, glycogen, and polyhydroxyalkanoates.

Polyhydroxyalkanoates are the only linear polyesters biodegradable and biocompatible with numerous applications in medicine, pharmacy (implants, covering of pharmaceuticals), and packaging. These thermoplastic polymers are accumulated as intracellular granules by different bacteria as carbon and energy storage material under conditions of restricted growth.[6] More than 40 different hydroxyalkanoic acids (HA) have been detected as constituents of PHA, but only few homopolyesters beside poly(3-hydroxybutyrate) (PHB) are available from bacteria.[7] Depending on the type of microorganism and substrate fed, they may produce polymers with different composition. It was shown that PHB copolymers that contained also an amount of 3-hydroxyvalerate (HV), named P(HB-co-HV), could be formed by cosubstrate feeding.[8]

Strains such as *Alcaligenes eutrophus* and *Pseudomonas oleovorans* have a high yield of production of this biopolymer. PHB homopolymer and copolymers of 3-hydroxybutyrate (HB) are currently being industrially produced by Zeneca under the trade name of Biopol. A mutant strain of *A. eutrophus* and glucose and propionic acid as substrates are used.[8] The copolymers have improved mechanical properties, making them suitable to replace petrochemical-based bulk plastics.[9] The improvement of the physical properties of the copolymers has been achieved by controlling the molar ratio HV/HB. The presence of HV repeating units in the chains decreases the hardness, crystallinity, and melting point of the polymer, thereby increasing its impact strength and processability. The carbon chain length of the substrate determines the range of monomer units incorporated into PHA. The 3-hydroxyacid formed possesses the same carbon chain length as the substrate.[6,8] Molecular chains can range from 600–35,000 repeating units.

In biological phosphorus removal processes, the synthesized PHA are, from this point of view, a byproduct. In the present work, the PHA accumulated in biological phosphorus removal from activated sludge are polyesters P(HB-co-HV). Much attention has been devoted to the study of homopolymers but heteropolymers seem to be more promising. Aiming to understand and characterize the polymers produced in the biological phosphorus removal process using different carbon sources, the anaerobic phase was studied. Acetate, propionate, and butyrate were used as single substrates or mixed with increasing overall concentration. The polymers formed were analyzed during the time course of the anaerobic period to determine their composition, average molecular weight ($\bar{M}_w$), and polydispersity ($\bar{M}_w/\bar{M}_n$, $\bar{M}_n$ are the number average molecular weight).

Materials and methods

Growth conditions

Medium. As otherwise stated, the medium used had the following composition (gl^{-1}): NH_4Cl, 0.16; $MgSO_4$, 0.6; $CaCl_2 \cdot 2H_2O$, 0.07; EDTA, 0.1; K_2HPO_4, 0.0923; KH_2PO_4, 0.0449; $CH_3COONa \cdot 3H_2O$, 0.6799; propionic acid, 0.2115; butyric acid, 0.1761; mineral solution, 2 ml ($FeCl_3 \cdot 6H_2O$, 1.5; H_3BO_3, 0.15; $CuSO_4 \cdot 5\ H_2O$, 0.03; KI, 0.03; $MnCl_2 \cdot 4H_2O$, 0.12; $Na_2MoO_4 \cdot 2H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.12; $CoCl_2 \cdot 6H_2O$, 0.15). Prior to autoclaving, the pH medium was set to 7.2 with the addition of 5 M NaOH.

Inoculum reactor. A sequenced batch reactor was inoculated with activated sludge from the wastewater treatment plant of Beirolas (Lisbon). The 2 reactor used with a operating volume of 1.5 l was operated under temperature and pH control (25°C and pH 7.0) and stirring (250 rpm). The sludge was exposed daily to three consecutive cycles. Each cycle was comprised of an 8 h period: anaerobiosis (2 h), aerobiosis (4 h), and settling (1 h). After

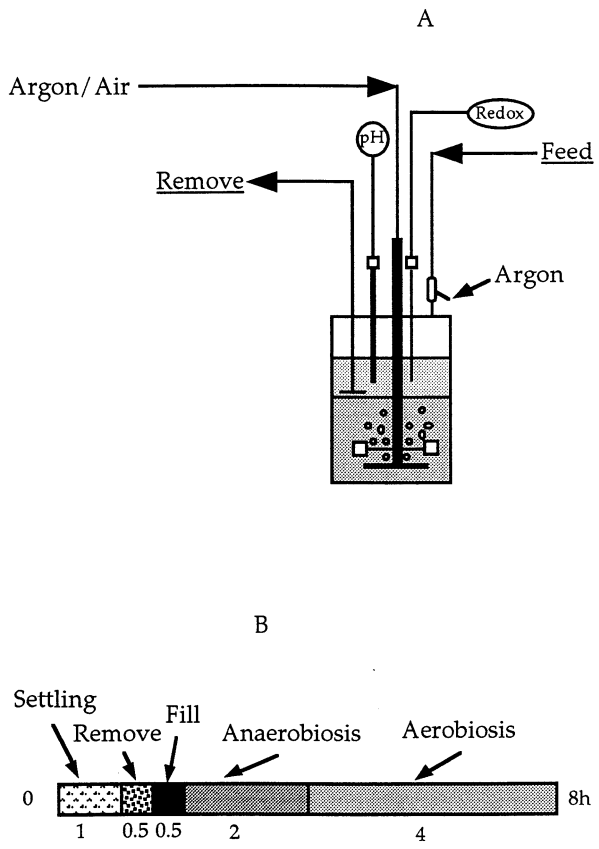

Figure 2 Sequence batch reactor (A) and cycle of operation (B)

settling, one third of the supernatant reactor volume was replaced with fresh medium (1 h) (*Figure 2*). The gas sparging rate was 0.2 vvm using argon during the anaerobic period and air during the aerobic period. A sludge retention time of 10 days was achieved by purging sludge daily at the end of the aerobic period.

Batch reactor. A defined volume of activated sludge was removed from the inoculum reactor, centrifuged, and resuspended in the same volume of fresh medium containing one third of phosphate concentration present on the medium used for the inoculum reactor. Acetate, propionate, and butyrate were added in variable concentrations (in terms of chemical oxygen demand-COD) and used as carbon sources. Unless otherwise stated, anaerobic conditions were provided using argon as sparging gas at 0.2 vvm. Temperature, pH, and stirring speed were the same used for the inoculum reactor.

Analytical methods

Cell dry weight determination. The quantification of cell dry weight was performed using the volatile suspended solids (VSS) technique according to Standard Methods.[10] Culture (5 ml) was filtered through a Whatman GF/C fiberglass filter (0.45 μm) previously dried and weighed. The filtrate was dried at 100°C for at least 24 h until constant weight. Finally, the culture filtrate was calcinated for 2 h at 550°C and weighed. The difference between these last two weights is expressed as VSS.

Carbon substrates. Organic acids determination was done by HPLC using a Shodex SH1011 column with 0.01 M sulfuric acid as eluent with an elution rate of 1 ml min^{-1} and an operating temperature of 50°C. A UV detector set at 210 nm (Merck Darmstadt, Germany) was used. Prior to injection, samples were filtered using a 0.2 μm membrane. The organic acid concentration obtained by HPLC was converted to COD using the oxidation stoichiometry for each acid.

Polyhydroxyalkanoate quantification. Polyhydroxyalkanoates determination was performed according to Braunegg *et al.*[11] and Comeau *et al.*[12] with minor modifications introduced by Satoh.[13] Culture (2 ml) was centrifuged and the pellet obtained was resuspended in 1 ml acidic methanol (20% H_2SO_4) with 0.5 mg ml^{-1} benzoic acid as internal standard. To this mixture, 1 ml chloroform was added and the solution was kept in a thermoblock at 100°C for 3.5 h. After cooling, 0.5 ml water was used for extraction. The chloroform phase was collected and molecular sieves (0.3 nm) were added for water retention. The equipment used was a gas chromatograph (Chrompack) with a flame ionization detector set to 220°C. The chloroform phase obtained (1 μl) was injected on-column in a 25 m × 0.25 μm Chrompack CPSIL-5CB column. Helium was used as the carrier gas. The temperature program for the analyses was: 1 min at 40°C; 30°C min^{-1} until 50°C; after 2 min of program start, the oven temperature was increased by 8°C min^{-1} until 160°C; and finally 1 min at 160°C (total time = 16.75 min). The injection port follows the temperature program for the oven.

A calibration curve using a PHB/HV standard (70%/30%; Merck) was obtained. Nine different concentrations of the standard were subjected to the same procedure used for the samples and injected on the GC. A direct correlation between HB concentration and corresponding peak area and also between HV concentration and corresponding peak area were obtained.

Tests were performed in order to determine if the time of acidic methanolysis reaction could affect the recovery of PHA. The best time for the recovery of the polymer was selected as 3.5 h.

Molecular weight determination of polyhydroxyalkanoates. For *polymer extraction*, 2 ml of culture was lyophilized. The sample was extracted with the same volume of chloroform by powerful stirring for 20 h. After filtration through a 0.2 μm fiberglass filter, the sample was dried under an inert gas flow followed by drying in a vacuum dessicator until constant weight. The polymer obtained was redissolved in chloroform on a final concentration of 0.2% (w/v). Before injection, the polymer solution was filtered using a 0.2 μm membrane filter.

For *polymer characterization*, the average molecular weights were determined using a gel permeation chromatography/size exclusion chromatography (GPC/SEC) apparatus (Waters, Milford, MA) including a solvent delivery system composed of a model 510 pump, a U6K injector, and a mode 401 refractive index detector. The operating temperature was 30°C using chloroform as eluent. A series of three Waters Ultrastyragel columns, 10^3Å, 10^4Å, and 10^6Å was used. Universal calibration was performed and the calibration curve was generated with monodisperse polystyrene (PS) standards (in the range 2×10^3 to 4×10^6 from Waters and Polymer Laboratories). The calibration curve was transformed using the Mark-Houwink relationship $[\eta] = K M^a$, where $[\eta]$ is the viscosity number limit and K and a are the Mark-Houwink constants, for each system polymer/solvent/temperature. The values of these constants used for the pairs PHB/chloroform and PS/chloroform were $K = 0.0118$ ml g^{-1}; $a = 0.78$ and $K = 0.0049$ ml g^{-1}; $a = 0.78$,[14] respectively. Sample injection volumes of 150 μl were used.

The majority of the molecular weight determinations were conducted in the initial period of all experiments since the most significant variations were expected to occur while the carbon source was being consumed.

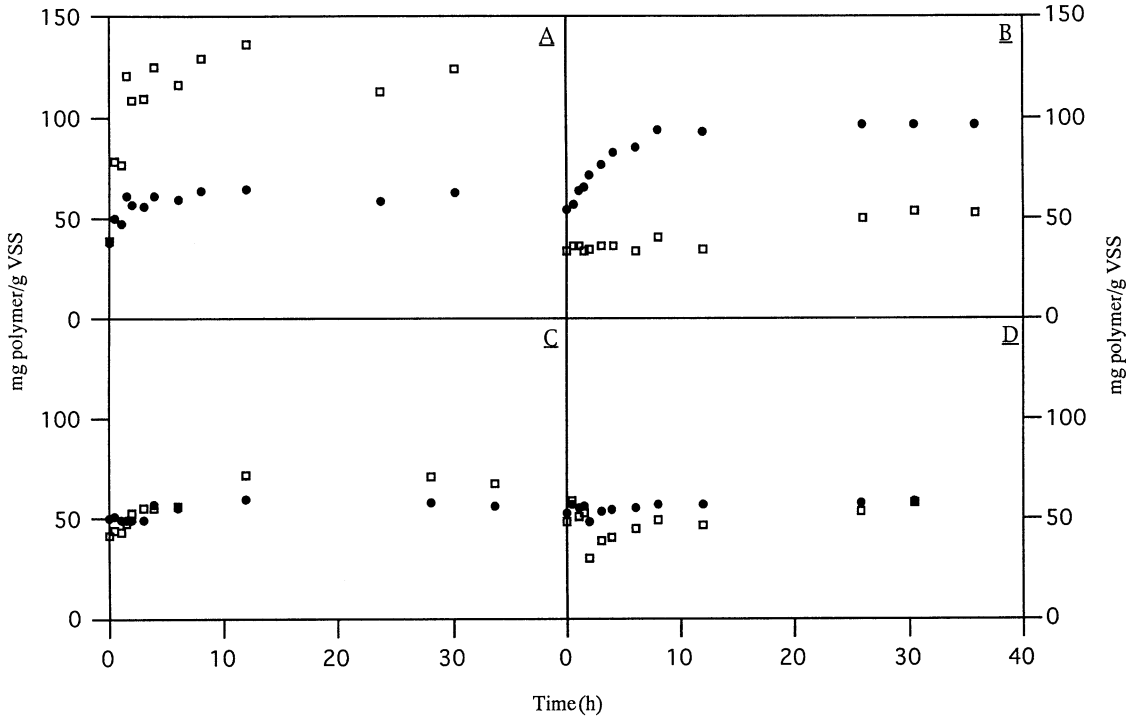

Figure 3 Polyhydroxyalkanoates *HB units* (□) *and HV units* (●) produced during anaerobic period using as carbon source, acetate (A), propionate (B), butyrate (C), and no carbon added (D)

Results and discussion

Effect of carbon source

The production of polyhydroxyalkanoates in the biological phosphorus removal process takes place during the anaerobic period. Since polyphosphate-accumulating bacteria grow under aerobic conditions, it can be considered that cell concentration remains approximately constant (between 2.5–3 g VSS l^{-1}) during the time course of the experiments. In order to evaluate the effect of different carbon sources on phosphate release and polyhydroxyalkanoate production, a fraction of sludge from the inoculum reactor was treated as described above and submitted to anaerobiosis during 40 h. Three different carbon substrates were used independently (acetate, propionate, and butyrate). A control reactor was also used without a carbon source addition.

The uptake of acetate leads to the best PHA production while butyrate contributes to the lowest polymer formation (*Figure 3*). Polymer composition is also affected by the carbon source used (*Table 1*). In fact, acetate consumption leads to the production of a copolymer of HB and HV repeating units with HB being predominant (75.25%). While using propionate, the opposite behavior occurred, i.e., the HV units are mainly produced (71.95%). The total amount of polymer produced from butyrate was minor when compared with the other two substrates, with HB slightly higher than HV. In the control experiment, no net production of HB or HV units was observed. Comeau *et al.*[15] reported approximately the same values of production of HB when using acetate (77%) and butyrate (52%) as carbon substrates but when using propionate, the obtained value was much lower (1%). Matsuo *et al.*[16] observed that when acetate was used as carbon source, 87% of the PHA produced were HB, 11% were HV, and the remaining 2% was 3-hydroxy-2-methylbutyrate (3H2MB) and 1% 3-hydroxy-2-methylvalerate (3H2MV). The same author with propionate as carbon source obtained 3% HB with the rest as HV (43%), 3H2MV (50%), and 3H2MB (6%).

Figure 4 shows the evolution of carbon concentration for the three substrates used. All substrates are completely consumed although at different rates. The higher substrate consumption rate was observed for acetate followed by propionate and finally butyrate.

The yield of polymer produced per carbon consumed

Table 1 Molar ratio (HB/HV) and molar percentage (HB/HV) obtained for the experiments using acetate, propionate, and butyrate independently or using 320, 960, or 1,920 mg COD l^{-1} of a mixture of the three carbon sources

Experiment	Molar ratio (HB/HV)	Molar percentage	
		HB	HV
Single substrate			
Acetate	3.04	75.25	24.75
Propionate	0.39	28.06	71.94
Butyrate	1.48	59.68	40.32
Mixed substrate			
320 mg COD	1.24	55.36	44.64
960 mg COD	0.47	31.97	68.03
1,920 mg COD	0.35	25.93	74.07

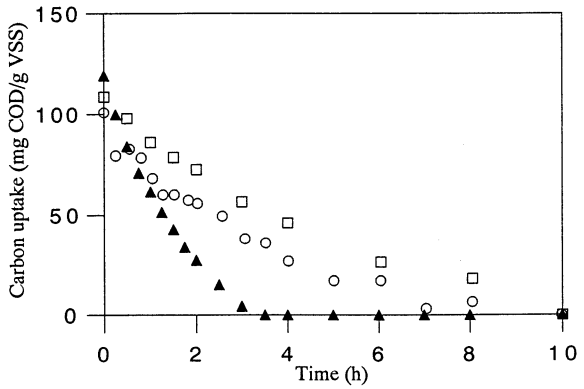

Figure 4 Carbon uptake during the anaerobic period when acetate (▲), propionate (○), and butyrate (□) were used independently

($Y_{P/S}$: mg polymer/mgCOD) was calculated plotting polymer formed against substrate consumed, linearizing, and taking the slope of the linear regression. A good correlation was obtained in all cases ($r = 0.99$). The $Y_{P/S}$ values obtained when using acetate, propionate, and butyrate were 0.97, 0.61, and 0.21, respectively. These results show that in this system, acetate is the best substrate for PHA production by the phosphorus-accumulating bacteria.

Samples from each batch culture were taken in order to determine the average molecular weight and polydispersity of the polymer produced. All samples analyzed exhibit several bands corresponding to different molecular weights. The evolution of the average molecular weight of the most representative band is presented in *Figure 5* for each substrate. At the end of the experiment, for all cases the emerging of fringes of much smaller molecular weight was observed, indicating degradation of the polymer (data not shown).

All polymers harvested are quite homogeneous since their polydispersity $\bar{M}_w/\bar{M}_n$ values presented a variation in the range 1–6 during the time course of the experiments. Since all the GPC chromatograms are unimodal, the polymers obtained are copolymers.[17] The polymers produced when acetate or propionate were used as carbon sources did not present a large variation in the average molecular weight during the time course of the experiments. The synthesized polymer from acetate has an average molecular weight close to 6×10^5 which is the value obtained for commercial polymers of this kind produced by *Pseudomonas* sp.; however, after the first hours of experimentation, a slight decrease in this value can be observed. Using butyrate as carbon source, the microorganisms seem to produce the highest molecular weight polymer although its concentration is too low. For this substrate, an increase in the average molecular weight was observed during the first 10 h of experimentation. After this period, the average molecular weight stabilizes.

Figures 3 and *5* are not discordant. The GC determination gives the total amount of units present in the sample. The GPC determination presents how many units are linked together (expressed in terms of mean molecular weight). In the case of the control experiment and at the end of the other three experiments, the long polymers were broken down to smaller ones (*Figure 5*) even though the total amount of the

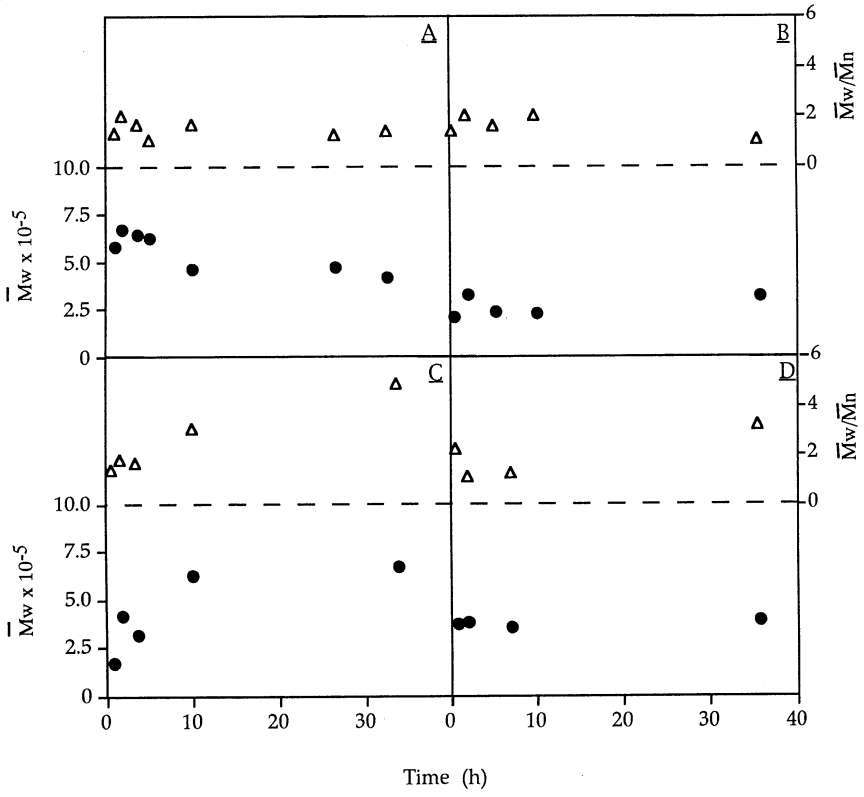

Figure 5 Average molecular weight $\bar{M}_w$ (●) and polydispersity $\bar{M}_w/\bar{M}_n$ (△) of the polymers produced during the anaerobic period using as carbon source acetate (A); propionate (B); butyrate (C); and no carbon added (D)

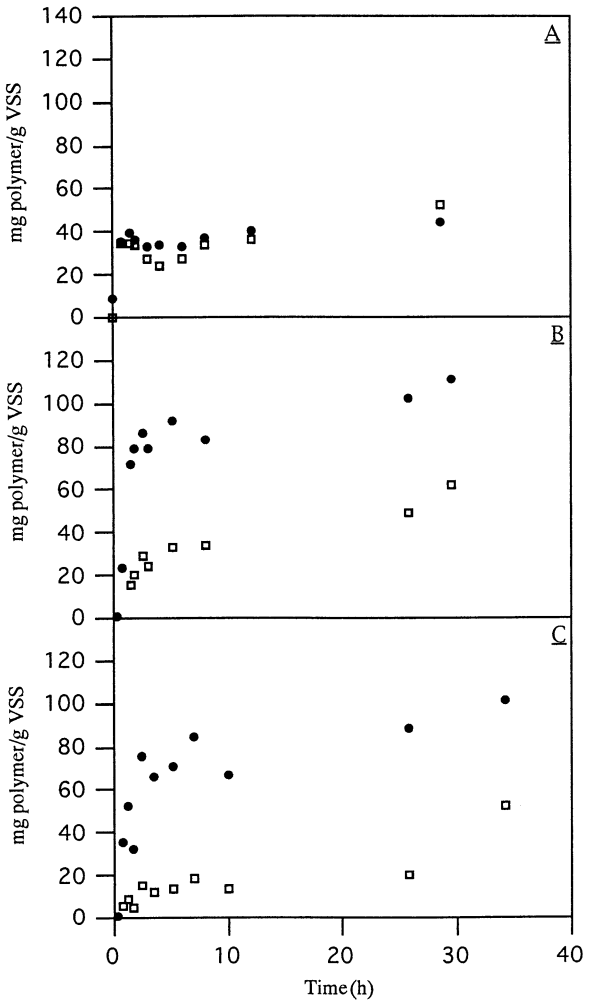

Figure 6 Polyhydroxyalkanoates *HB units* (□) *and HV units* (●) produced during the anaerobic period using as carbon source 320 COD (A); 960 COD (B) or 1,920 COD (C)

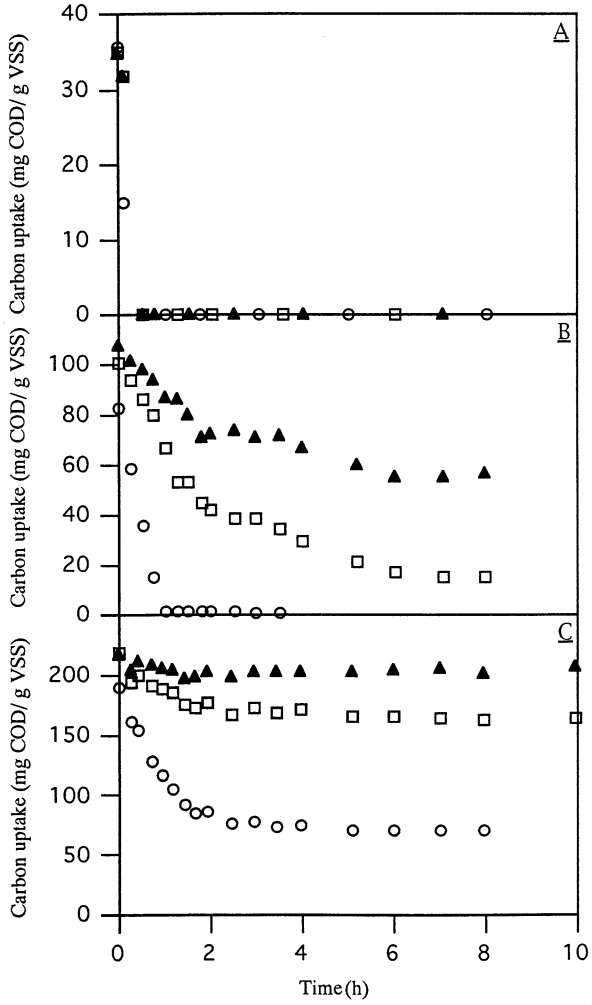

Figure 7 Carbon uptake during the anaerobic period when 320 COD (A); 960 COD (B); or 1,920 COD (C) were used with acetate (▲); propionate (○); and butyrate (□)

polymer is remaining approximately the same (*Figure 3*). The total amount of the most representative band decreased although its mean molecular weight remained the same. The energy obtained from the polymer hydrolysis may be used for microorganism maintenance.

Effect of using a mixed carbon source at different concentrations

Aiming to understand if the carbon concentration could influence the production of PHA by phosphate-accumulating bacteria, a mixture of acetate, propionate, and butyrate with the same concentration in terms of chemical oxygen demand (COD) was used. The total value for COD was 320 mg O_2 l^{-1} in a reference experiment (the same value previously used during studies on the effect of carbon source). Experiments using three and six times this value (960 mg O_2 l^{-1} and 1,920 mg O_2 l^{-1}, respectively) were also performed. Results are shown in *Figure 6* and *Table 1*.

For a COD concentration of 320 mg O_2 l^{-1} the polymer contains almost the same amount of HB (55.36%) and HV (44.64%) units. Increasing the carbon concentration to three and six times the reference amount, it was observed that the microorganisms produce more HV than HB units (*Table 1*), the highest polymer production being obtained during the 960 mg O_2 l^{-1} experiment.

These results can be understood if the carbon consumption is considered (*Figure 7*). It was observed that among the three substrates present in the medium, propionate was always the first to be exhausted followed by butyrate and lastly acetate. These results are in contrast with the observed rates for the three substrates independently (*Figure 4*) but they were obtained systematically in repeated experiments. In the reference experiment, the carbon substrates are readily consumed. When increasing the carbon concentration three times, it takes about 8 h to halt consumption. Using six times the reference concentration, a large fraction of the substrate is left over (including propionate). Since propionate mainly originates HV units, this explains the polymer composition on HV being much higher in the 960 and 1,920 mg O_2 l^{-1} experiments. For the highest substrate concentration, acetate is only slightly consumed and so less

Table 2 Experimental and balanced $Y_{P/S}$ values obtained using as carbon source 320, 960, or 1,920 mg COD l^{-1} of a mixture of three carbon sources

Experiment	Total COD cons. (mg COD g^{-1} VSS)	% COD consumed			$Y_{P/S}$ (exp.)	$Y'_{P/S}$ (balanced)
		Acet.	Prop.	But.		
320 COD	106.60	33.3	33.3	33.3	0.58	0.60
960 COD	223.62	14.3	47.7	38.0	0.52	0.51
1,920 COD	218.68	7.4	67.0	25.6	0.49	0.53

HB units are produced in comparison with the triplicate concentration assay.

The $Y_{P/S}$ values determined for the three experiments were calculated using data until the carbon consumption stops (*Table 2*). The amount of propionate consumed was always higher than the uptake of acetate and butyrate. Taking into consideration that the values of $Y_{P/S}$ calculated for each substrate independently (previous results) decreases from acetate to propionate to butyrate, the results accomplished with mixed substrates can be explained. In fact, using the $Y_{P/S}$ value obtained for each substrate independently and multiplying it by the percentage of COD taken up for that substrate, the contribution of that specific substrate to the overall polymer production is obtained. Adding the three yield values determined by this way, a balanced $Y'_{P/S}$ value for each experiment is obtained. Comparing the $Y_{P/S}$ values calculated from the mixed substrate experiments with the balanced $Y'_{P/S}$ values, it can be observed that they are quite similar. This fact demonstrates that the amount of polymer produced when a mixture of substrates is used is the sum of the individual contributions of each substrate *per se*.

If the carbon consumption is considered (*Figure 7* and *Table 2*), we would expect that $\bar{M}_w$ would be higher in experiments using 320 mg COD l^{-1} than in the last two assays since the amount of acetate consumed is higher. As observed before, the average molecular weight of the most representative fraction is higher when HB is present in a considerable amount (compare *Figures 6* and *8*). That is true for the 320 mg COD l^{-1} and for the 1,920 mg COD l^{-1} experiments; moreover, this fact can explain the lower average molecular weight values obtained for the 1,920 mg COD l^{-1} experiment. Due to the scattered data in the experiment using 960 mg COD l^{-1}, this conclusion cannot be clearly withdrawn. For 320 mg of COD, the $\bar{M}_w$ value decreases clearly at the end of the assay. This behavior can be explained since carbon is consumed so abruptly that the microorganisms need to use their reserves for maintenance and, for this reason, at approximately 35 h the polymer obtained has a $\bar{M}_w$ value below 2.5×10^5. Once again, polymer was broken into smaller fractions with the surge of fringes with much lower average molecular weights.

An additional experiment using a COD concentration of 320 mg O_2 l^{-1} in a mixture containing acetate, propionate, and butyrate was accomplished using an anaerobic followed by an aerobic period. The length of both periods were 2 and 4 h, respectively. Both HB and HV units were produced in similar amounts (*Figure 9A*). As was expected by metabolic considerations, PHA produced during anaerobiosis was consumed during the following aerobic period. Even though the data are scattered, *Figure 9B* shows that there is an increase in average molecular weight during the anaerobic period and a decrease during aerobiosis, thus for PHA

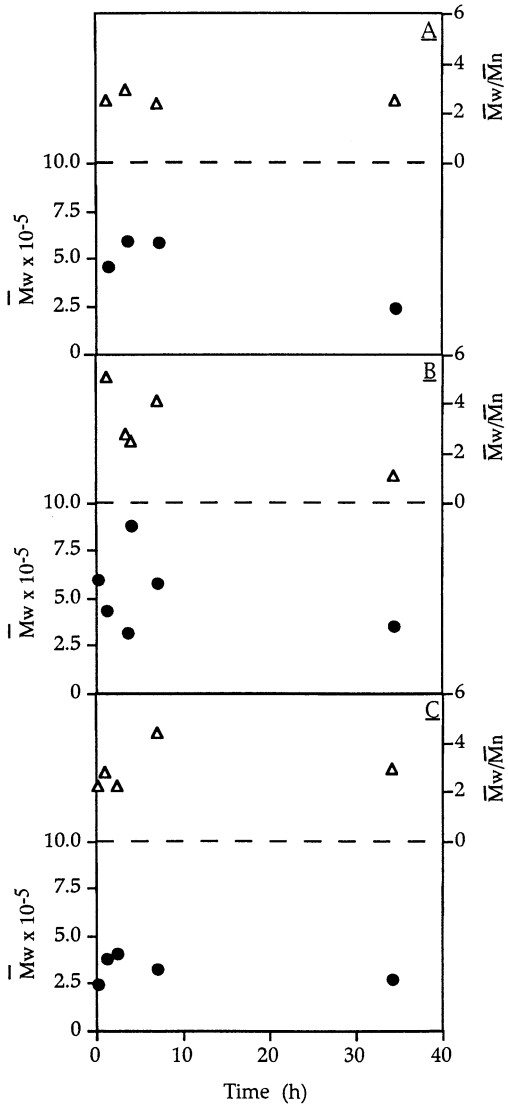

Figure 8 Average molecular weight $\bar{M}_w$ (●) and polydispersity $\bar{M}_w/\bar{M}_n$ (△) of the polymers produced during the anaerobic period using as carbon source 320 COD (A); 960 COD (B); or 1,920 COD (C)

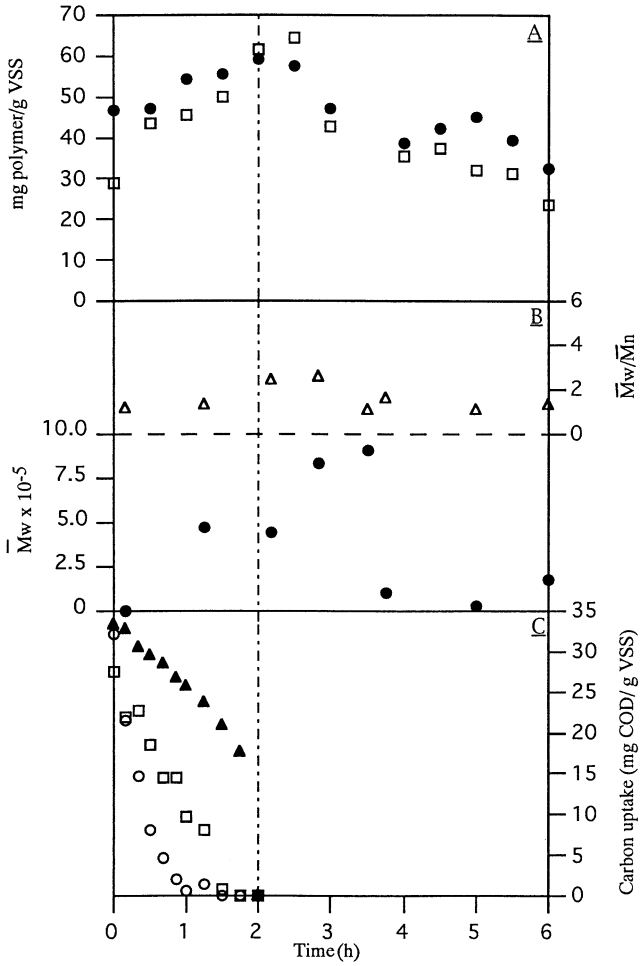

Figure 9 Anaerobic and aerobic periods for a reactor fed with a mixture of acetate, propionate, and butyrate in a total amount of 320 COD. Polyhydroxyalkanoates: HB units (□) and HV units (●) (A) average molecular weight $\bar{M}_w$ (●) and polydispersity of polymers $\bar{M}_w/\bar{M}_n$ (△) (B), carbon uptake: acetate (▲); propionate (○); and butyrate (□) (C)

production and recovery, the aerobic period is crucial. Cells should be harvested not immediately at the end of the anaerobic period, but at a time when a good relation between production and average molecular weight of the polymer produced is obtained since both maximums do not seem to be exactly simultaneous (*Figures 9A* and *B*). The carbon consumption pattern can be observed very neatly with propionate the first to be completely consumed followed by butyrate and finally acetate (*Figure 9C*). When the carbon uptake for this experiment is compared with the one observed in *Figure 7A*, it can be seen that there was a faster consumption on *Figure 9C*. This fact can be understood if the initial concentration of PHA present is considered. For the experiment using 320 mg COD l^{-1}, there was no PHA present at the beginning of the experiment. On the contrary, an appreciable amount of polymer was detected in the beginning of the experiment present in *Figure 9A*. This behavior suggests that the microorganisms involved more readily take up external carbon substrates when they had no PHA accumulated (being the cells in starvation).

Comparison with PHA polymers produced by pure cultures (literature data)

Even though most of the research work has been focused on homopolymers, heteropolymers are more interesting when their mechanical and physical properties are considered. The molecular weight depends strongly on the bacterial strain and the carbon source used. Concerning the literature available, *Table 3* lists values of $\bar{M}_w$ and polydispersity of homo- and copolymers obtained from pure cultures of microorganisms.

Most of the values observed in the literature for $\bar{M}_w$ are not absolute values. In general, they refer to the polystyrene standards curve. The values presented in this work were corrected for PHB/chloroform using the Mark-Houwink constants with these values being absolute ones.

Comparing the values of $\bar{M}_w$ and $\bar{M}_w/\bar{M}_n$ shown in *Table 3* with the corresponding values obtained in this work, it can be concluded that copolymers obtained with the mixed culture used in this study are very similar to those obtained with pure cultures. In the present work, most of the copolymers were identical to those obtained with *Pseudomonas* strains with values of $\bar{M}_w$ close to 6×10^5; nevertheless, the existence of residual fractions with decreasing molecular weight along the biological process was observed experimentally. In some cases, fractions of higher $\bar{M}_w$, around $1–2 \times 10^6$, were produced. This is similar to the polymer produced by *Alcaligenes* sp. (*Table 3*). The literature reports the variation in $\bar{M}_w$ for PHB during batch fermentation of *A. eutrophus* which is explained in terms of simultaneous synthesis and degradation of the polymer or synthesis of lower $\bar{M}_w$ during the later stages of polymer accumulation.[6,19,29]

Conclusions

This work is integrated in a more extended study whose main goal was to optimize phosphorus removal in a wastewater treatment process with polymer production a side stream subject. The production of PHA by biological phosphorus removal sludge may be an inexpensive way of producing a highly increased value product and also a way to valorize excess sludge.

The carbon source fed to the microbial culture very widely influences not only the composition but also the average molecular weight of the polymers produced. From a practical point of view, if the goal is to remove phosphorus from wastewater, acetate leads to the best process performance since it conducts to a higher yield of polymer produced per COD unit consumed. The average molecular weight of the polymer obtained was of the same order of magnitude as the polymers produced commercially from pure cultures of microorganisms. The use of propionate should be considered if a higher relative amount of HV units are required. Butyrate was expected to be a good substrate since its carbon skeleton is quite identical to 3-hydroxybutyrate, the precursor of PHB; however, the $Y_{P/S}$ yield obtained is quite low even though the polymer produced has a higher average molecular weight.

The amount of carbon source fed, and in particular, the type of carbon source used is of extreme importance. When

Table 3 Values of $\bar{M}_w$ and polydispersity of PHA obtained from pure cultures of bacteria

Microorganism	Type of PHA	Substrate	$\bar{M}_w \times 10^{-5}$	$\bar{M}_w/\bar{M}_n$	Reference
Alcaligenes eutrophus	PHB	N-alkanoates of C_2–C_{22}	2.3–15.5	1.4–2.5	18
	P(3HB-co-4HV)	N-alkanoates of C_2–C_{22}	0.36–30	1.7–2.5	18
	PHB	Butyric acid	11.3–11.6	2.0–4.2	19
	PHB	Butyric acid	12–33	2.3–7.7	20
	P(3HB-co-4HV)	γ-Butyrolactone and butyric acid	4.3–7.8	1.8–2.7	21
	P(3HB-co-4HV)	Butyric and valeric acids	12.2	1.7	19
	PHB	Glucose	9.0–20	2.0–5.0	22
	PHB	Glucose	21–23	3.5	23
	PHB	Radiolabelled glucose	13–20	3.0–4.1	24
Alcaligenes latus	P(3HB-co-4HV)	Sucrose and γ-butyrolactone	2.9–3.9	1.5–2.0	25
Azotobacter vinelandii	PHB	Glucose and fish peptone	17–28	1.7–3.0	26
	PHB	Beet molasses	10–45	1.2–1.8	27
Methylobacterium extorquens	PHB	Methanol	2.0–6.0	4.3–5.0	28
(*Pseudomonas* sp. AM1)	PHB	Methanol	1.7–6.2	4.0–8.3	29
	PHB	Methanol and ethanol	3.2	4.2–5.7	29
	PHB	Methanol and glycerol	2.6–3.9	3.3–4.5	29
	PHB	Methanol and fructose	11.1–11.3	2.9–3.0	29
	PHB	Sodium succinate	7.2–16.6	2.9–5.7	29
	PHB	Sodium succinate	9.0–17.0	2.9–5.7	28
Pseudomonas 135	PHB	Methanol	2.6–3.7	10.4–11.1	30
Pseudomonas cepacia	PHB	Glucose, xylose and lactose	2.2–8.7[a]	1.6–3.9	31
Pseudomonas oleovorans	PHA and copoly(HA) from PH hexanoate to PH decanoate	N-alkanoic acids from formate to decanoate	0.92–3.7	2.0–2.5	32
	PHB	*n*-Octane and *n*-octene	1.9–2.6	1.5–2.3	33
Rhodospirillum rubrum	P(3HB-co-HV-co-HPE)[b]	4-pentenoic and pentenoic acids	3.4–4.8	3.1	34

[a]Absolute values of $\bar{M}_w$ obtained with universal calibration
[b]HPE: 3-hydroxy-4-pentanoate

using mixed substrates, the $Y_{P/S}$ value is the sum of the individual contributions of each substrate. With a carbon concentration of 320 COD, identical amounts of HB and HV units were produced. The utilization of more concentrated carbon substrates leads to the consumption of propionate primarily, the P(HB-co-HV) being enriched in HV units.

Acknowledgments

The authors acknowledge the financial support of Junta Nacional de Investigação Científica (JNICT)/Direcção Geral da Qualidade do Ambiente (DGQA) under project n° PEAM/P/APR/107/91 and PEAM/C/SEL/494/95 (JNICT). P. C. Lemos acknowledges JNICT for grant BD/1217/91-IF and C. Viana acknowledges Program PEDIP for grant II/f5/1691. The authors would also like to thank the facilities for collecting activated sludge at the wastewater treatment plant of Beirolas (Lisbon).

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