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Production of poly-β-hydroxybutyrate from methanol: characterization of a new isolate of *Methylobacterium extorquens**

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Summary. Poly-β-hydroxybutyric acid (PHB) and similar bacterial polyesters are promising candidates for the development of environment-friendly, totally biodegradable plastics. The use of methanol, one of the cheapest noble substrates available, may help to reduce the cost of producing such bioplastics. As a first step, a culture collection of 118 putative methylotrophic microorganisms was obtained from various soil samples without any laboratory enrichment step to favour culture diversity. The most promising culture was selected based on rapidity of growth and PHB accumulation and later identified as *Methylobacterium extorquens*. This isolate was obtained from soil contaminated regularly with used oil products for some 40 years. Concentrations of methanol greater than 8 g/l affected growth significantly and the methanol concentration was optimal at 1.7 g/l. PHB concentrations averaged 25–30% (w/v) of dry weight under non-optimized conditions. Controlling methanol concentration, using an open-loop configuration, led to biomass levels of 9–10 g/l containing 30–33% PHB while preventing methanol accumulation. The new isolate was also able to produce the co-polymer PHB/poly-β-hydroxyvalerate (PHV) using the mixture methanol + valerate. The PHV-to-PHB ratio was about 0.2 at the end of the fermentation. An average molecular mass varying between 2 and 3×10^5 Da was obtained for three PHB samples using two different measurement methods.

Introduction

It is well known that many bacteria may accumulate in their cytoplasm granules of biopolymers with thermoplastic properties similar to those of polypropylene (Byrom 1987; Dawes 1988; Lafferty et al. 1988). These biopolymers, of the polyester type, belong to the class of poly-β-hydroxyalkanoates (PHA), of which poly-β-hydroxybutyrate (PHB) is the best known. These polyester biopolymers are biodegradable and could therefore be used in the manufacture of some plastics. However, the present high cost of PHB and related biopolymers is a severe deterrent to their use on a large scale. Substrate and recovery costs are the two major factors responsible for the high cost of these polymers. Methanol, one of the cheapest noble substrates available (Byrom 1987; Large and Bamforth 1988; Dijkhuizen et al. 1985), could be favourably used to help reduce production costs.

In this paper, we report on the isolation and initial characterization of a new soil isolate of *Methylobacterium extorquens*, a methylotrophic bacterium able to accumulate significant amounts of PHB during growth on methanol. The isolate was also able to produce the co-polymer PHB/poly-β-hydroxyvalerate (PHV). Part of this work was presented at the 1990 Annual Meeting of the Society for Industrial Microbiology, Orlando, Florida, USA, P40.

Materials and methods

Media

Medium 784 (American Type Culture Collection 1985) was used with modifications, if necessary, described in the text. All glass redistilled methanol was used without sterilization as the sole or main carbon substrate. For several experiments, HySoy, a complex organic nitrogen source (Sheffield Products, Norwich, N. Y., USA), was also added to Medium 784.

Isolation of methylotrophic microorganisms

For each soil sample (six samples, Table 1) about 10 g soil was added to 100 ml sterile saline (0.85% NaCl) and the mixture vigorously shaken by hand. Each sample was then serially diluted in sterile saline and duplicate 1-ml aliquots of 10^{-4} to 10^{-8} dilutions spread on solid Medium 784 containing low ash agar (Agar Noble) at 15 g/l and 1% (w/v) methanol. The plates were incubated at 28–30° C for up to 11 days. Subsequent culture purification on minimal medium containing methanol, using regular purified agar

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Table 1. Origin and description of the soil samples used for the isolation of methylotrophic microorganisms

Soil sample	Origin/description
A	Rich garden soil
B	Oily, black, sandy soil subjected to frequent contamination by used oil products for at least 40 years
C	Very similar to B
D	Dark, rich garden soil used for the production of pumpkins
E	Very moist soil near firewood
F	Rich garden soil used for the production of beets

All soil samples from locations in the Quebec City area (Canada)

(Difco, Detroit, Mich., USA), was done as needed to obtain pure cultures.

Selection of the best PHB-producing microorganism

Each pure culture (118 in all) was examined by phase-contrast microscopy for the presence of intracellular refractile granules suggesting the presence of PHB. The 18 cultures testing positive were thereafter grown in minimal medium containing methanol and one-third of the regular amount of nitrogen (to favour PHB accumulation); methanol was added periodically during growth to enhance biomass and PHB production. Four cultures showed superior PHB production and were selected for final testing based on rapidity of growth and PHB production.

Toxicity of methanol

For these experiments, a series of 500-ml erlenmeyer flasks containing 200 ml medium, and various concentrations of methanol, was inoculated with a culture of the organism grown into the same medium (1% methanol, v/v) and incubated at 30° C at an agitation rate of 250 rpm. The medium contained (per litre): K_2HPO_4, 0.7 g; KH_2PO_4, 0.54 g; NH_4Cl, 0.5 g; HySoy, 5 g; $(NH_4)_2SO_4$, 1.25 g; methanol, 1% (v/v).

Fermentor studies

Fermentors. All fermentor studies were carried out using 3.5-l continuously stirred baffled fermentors (Chemap, Volketswil, Switzerland) having a working volume of 2.0–2.2 l and equipped with two disc-turbine impellers for agitation, pH and pO_2 electrodes (Ingold, Urdorf, Switzerland), a foam sensor and a mechanical foam breaker.

Control of methanol addition. In these fermentations, the microorganism was grown using a discontinuous fed-batch mode for methanol addition. Methanol was added discontinuously in order to maintain its concentration near the optimal value ($S_{optimal}$ calculated as 1.7 g/l). Methanol was first added in an exponential mode for the first 20–24 h, with the flow rate for methanol (F, h^{-1}) calculated according to the following equation:

$$D = \frac{F}{V} = \frac{\mu \cdot X_0 \cdot e^{\mu(t-t_0)}}{Y_{X/S} \cdot (S_0 - S)}$$

where D = dilution rate, F = feed rate, $Y_{X/S}$ = biomass yield (dry weight) on methanol, μ = the growth rate, X_0 = initial biomass concentration, S_0 = methanol concentration in the feed and S = methanol concentration in the fermentor. After 20–24 h, the flow rate for methanol was kept constant at the last calculated rate. Methanol addition was done each hour using a pump and a programmable timer. Every 8 h, 10 ml nutrient mixture was also added to favour growth: the mixture contained ammonium sulphate at 55 g/l and HySoy at 100 g/l.

Other analyses

Optical density. The optical density of the cultures was determined at 600 nm using 16-mm i. d. glass tubes and distilled water as the blank. All readings, after correcting for medium, were adjusted according to the tables of Toennies and Gallant (1949).

Dry weight. Samples of the cultures (5–20 ml) were filtered through a 0.45-μm membrane, the cells washed once with water, and the samples dried at 105° C to constant weight.

Sugars and ethanol. These substances were measured by HPLC using an organic acid column (Ion-300, 7.8 mm i. d. × 300 mm long; Interaction Chemicals, Mountain View, CA, USA). Other conditions were: temperature, 30° C; flow rate, 0.4 ml/min; mobile phase, .00173 M H_2SO_4. Detection was done using both a differential refractometer (model 410, Waters Scientific, Milford, Mass., USA) and a tunable absorbance detector (λ = 210 nm, model 484, Waters Scientific).

Methanol. Methanol concentration was determined by gas chromatography (GC) using a Perkin-Elmer (Norwalk, Conn., USA) chromatograph (Model Sigma 2000) equipped with a flame ionisation detector and a stainless steel column (HaySep Q column, 6 ft × 0.125 in. o. d., 80/100 mesh; Chromatographic Specialties, Brockville, Canada). Other conditions were: column temperature, 150° C; injector and detector temperature, 200° C; carrier gas, nitrogen (flow rate; 34.3 ml/min).

PHB and PHB/PHV. Analysis was performed by GC using the method of Braunegg et al. (1978). Analyses were done on samples of cell biomass stored at −20° C after centrifugation at 2000 *g* for 20 min. A glass column (6 ft × 4 mm i. d.), filled with 10% Carbowax 20M TPA on WAW-DMCS, 80/100 mesh, was employed for these analyses (Chromatographic Specialties).

Microbial identification. Identification of the selected microorganism was done using the following tests: oxidase test, done using Taxo N discs (BBL, Cockeyville, Md., USA); hydroxypyruvate reductase test, as described by Large and Quayle (1963) using a French press cell extract; fatty acid analysis, carried out by MicroCheck (Northfield, Vt., USA).

Molecular mass of PHB

Viscosimetric method. PHB was first extracted with chloroform according to Berger et al. (1989). The relative viscosimetric shear rate of solutions (in chloroform) containing increasing concentrations of PHB (0.025 to 0.5%, w/v) was determined at 30 ± 0.1° C using a rotational rheometer (Contraves, Zurich, Switzerland; model Low Shear 30). The shear rates used varied between 0 and 100 s^{-1}. The intrinsic viscosity [η] was calculated using the equation of Huggins as found in Marchessault et al. (1970). Molecular mass was calculated using the Mark-Houwink equation, and the values for the constants *K* and *a* were those of Akita et al. (1976) and Marchessault et al. (1970).

Gel permeation chromatography. As described in Berger et al. (1989), PHB samples (0.5%, w/w) were dissolved in chloroform.

Production of the co-polymer PHB/PHV

These experiments were done in fermentors starting with 2 l medium. The basal medium contained (per litre): K_2HPO_4, 0.7 g; KH_2PO_4, 0.54 g; NH_4Cl, 0.5 g; HySoy, 5.0 g; and $MgSO_4$, 0.11 g (pH after sterilization, about 6.8). Other conditions were: temperature, 30° C; pH maintained at 7.0 with 5 M KOH and 1 M H_2SO_4; dissolved oxygen level (pO_2) maintained at 65% air saturation; aeration set at 0.5 vvm; agitation rate, 350 rpm; inoculum size, 5% (v/v), using a culture growing on the same medium. Following total consumption of the methanol initially added, the production of the co-polymer PHB/PHV was induced by adding a mixture of methanol and valerate to the culture to give a final concentration of 1.0% (w/v) and 0.5% (w/v) for methanol and valerate, respectively. Methanol was subsequently added twice more in order to enhance co-polymer production and cell viability.

Results

Isolation of methylotrophic microorganisms

A collection of 118 putative methylotrophic microorganisms was obtained from the six soil samples. The soil samples were used directly, without any laboratory enrichment step, in order to favour microbial diversity. Low ash, highly purified agar (Agar Noble, Difco) was used in the initial steps to reduce the risks of isolating false methylotrophic microorganisms. Over 95% of the cultures were bacteria, the rest being probably yeast cultures and perhaps one fungal culture. The virtual absence of yeasts in the collection was noted with much surprise since several methylotrophic yeast species exist and no fungal antibiotic was included in the medium during isolation. The absence of methylotrophic fungi was less surprising as few of these have been isolated so far (Tani 1984). The soil samples contained at most between 10^6 and 10^7 methylotrophic microorganisms per gram of wet soil with the highest numbers obtained with samples contaminated with used oil products. Regular contamination with used oil products appeared to have led to some enrichment of methylotrophic bacteria.

Selection of the best PHB-producing bacterium, partial characterization and identification

Of the 118 cultures, 18 showed strong evidence for the presence of cytoplasmic PHB granules. Further testing for rapidity of growth and PHB production under partial limitation of nitrogen led to the selection of culture no. 58 as the most promising PHB producer. This bacterial isolate originated from soil sample C (Table 1). Initial characterization strongly suggested that culture no. 58, a pink-pigmented bacterium, belonged to species of *Methylobacterium*. The bacterium has a coccobacillus-to-rod shape, is Gram-negative, motile, tested positive for both catalase and oxidase activity, and possesses the enzyme hydroxypyruvate reductase indicating that it assimilates carbon via the serine pathway. Fatty acid analysis of the culture identified it as *M. extorquens*, with a similarity index of 0.627 indicating an excellent match. Pink-pigmented cultures constituted 44% of the collection and 83% of the 18 most promising cultures.

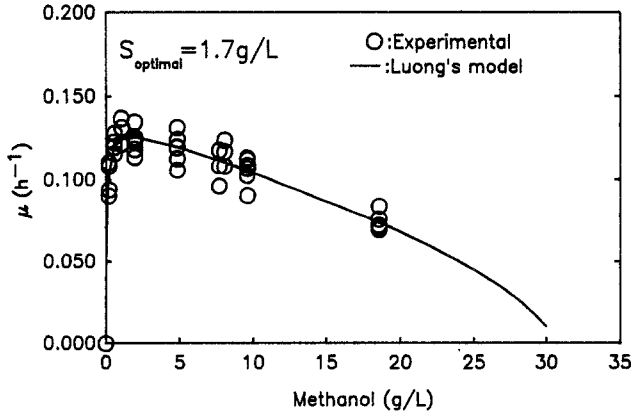

Fig. 1. Methanol toxicity: influence of initial methanol concentration on the instantaneous growth rate (μ) of *Methylobacterium extorquens*. The experiment was done in shake flasks using medium containing 0.5% HySoy as described in Materials and methods. The optimal methanol concentration ($S_{optimal}$) was calculated using Luong's model (1987)

Substrate utilization

The results from flask experiments (results not shown) indicated that this methylotrophic microorganism did not use glucose, lactose, or sucrose, but used ethanol to a certain extent. Medium acidification resulted from substrate utilization. Biochemical tests using Biolog plates for Gram-negative bacteria indicated that the bacterium was a poor utilizer of sugars but is mainly an organic acid utilizer since it used formic acid, β-hydroxybutyric acid, D,L-lactic acid, malonic acid, propionic acid, and succinic acid (results not shown).

Toxicity of methanol

The microorganism was grown in shake flasks at different initial methanol concentrations and instantaneous growth rates calculated (Fig. 1). Growth was significantly affected at methanol concentrations above 8 g/l. Using the mathematical model developed by Luong (1987), the optimal methanol concentration was estimated to be 1.7 g/l and the methanol concentration completely inhibiting growth was 30 g/l. For practical purposes, methanol concentrations up to 5 g/l may be used without appreciable deleterious effects on growth of the organism (Fig. 1).

Growth of the microorganism

The *M. extorquens* isolate grew relatively well in a minimal broth containing only various salts and methanol at 5–8 g/l. Nevertheless, growth was significantly im-

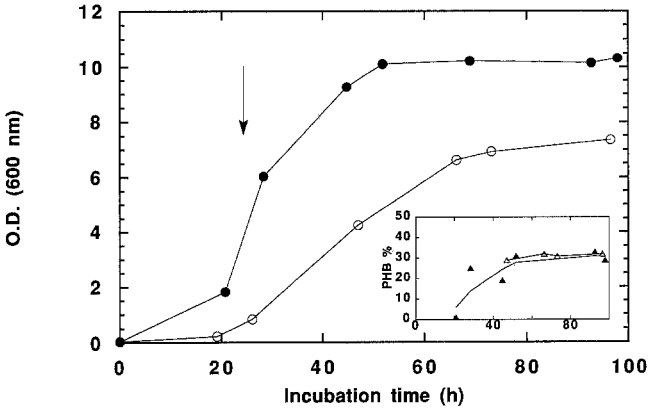

Fig. 2. Growth of *M. extorquens*, measured by optical density (O.D.), and poly-β-hydroxybutyrate (PHB) production in minimal or complex medium with methanol as sole or main substrate. Experiments were carried out in 3.5-l fermentors with a methanol concentration at 0 h of 1% (v/v). At about 24 h (*arrow*), methanol and ammonium sulphate were added periodically until the end of the fermentation. The pH was controlled at 6.5 ± 0.2 and the pO_2 maintained at 65% air saturation as described in Materials and methods: ○, △, minimal medium 784 containing NH_4Cl at 0.5 g/l as the only nitrogen source; ●, ▲, minimal medium 784, without the trace elements, containing 0.75% HySoy in addition to the regular amount of NH_4Cl. It was assumed that HySoy also supplied most of the trace elements present in the original medium 784

proved by enriching the minimal medium with a complex organic nitrogen source (HySoy) as shown in Fig. 2. PHB production was essentially identical in either the minimal or the enriched broth and averaged 30% (w/w) of dry weight without any deliberate optimization. Transmission electron micrographs of the cells, at two stages of the growth, indicated that PHB accumulation was negligible in cells in the early exponential phase but was significant in cells in the late stationary phase (results not shown).

Control of methanol concentration

Attempts were made to increase cell biomass and PHB production in small fermentors by controlling methanol concentration at its optimal value (1.7 g/l) to minimize both the toxic effects of methanol and methanol evaporation. A preliminary experiment was first done to evaluate the yield of cell biomass on methanol ($Y_{X/S}$): a value of 0.35 was obtained for a culture in the exponential phase of growth. Knowing $Y_{X/S}$ and the initial cell biomass concentration (X_0), and assuming that $Y_{X/S}$ was constant during exponential growth, methanol was added to the culture using a pump linked to a programmable timer. In one representative experiment (results not shown), methanol was added continuously, in an exponential mode, to the culture for the first 24 h, then added at a constant rate for the rest of the experiment. Using this inexpensive control system, cell biomass reached about 9 g/l (dry weight) at 160 h; the cells at this point contained about 30% (w/w) PHB. Simultaneous monitoring of the methanol concentration by GC indicated that methanol was limiting at some times during the fermentation.

Table 2. Molecular mass of poly-β-hydroxybutyric acid (PHB) samples produced from methanol by *Methylobacterium extorquens*

Sample	Molecular mass (Da)	
	GPC method	Viscosimetric method
A	217335	197000 ± 21000
B1	266835	265000 ± 52000
B2	235566	268000 ± 41000

PHB was produced in flasks, recovered and purified as described in Materials and methods: GPC, Gel permeation chromatography

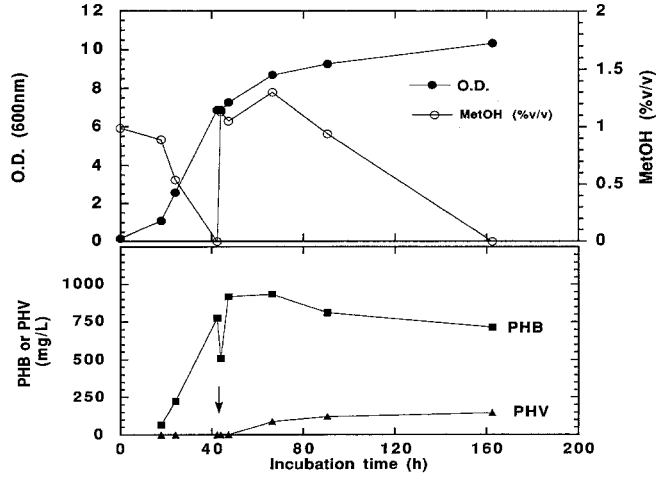

Fig. 3. Production of the co-polymer PHB/poly-β-hydroxyvalerate (PHV) by *M. extorquens*. Following growth on methanol in medium containing 0.5% HySoy, the addition (*arrow*) of a mixture of methanol (MetOH) and valerate (0.5%, w/v) to the culture resulted in the production of the co-polymer PHB/PHV. At the end of the fermentation, the PHV/PHB ratio was 0.2. See Materials and methods for details

Molecular mass of PHB

Three samples of PHB produced in flasks were used to estimate the average molecular mass of the PHB produced by *M. extorquens*. Molecular mass values were determined using two different methods: gel permeation chromatography (GPC) and viscosimetry (Table 2). Molecular mass values were in the 200–300 × 10^3 Da range using both methods.

Production of the co-polymer PHB/PHV

Experiments were performed to verify whether or not the new *M. extorquens* isolate could also produce the co-polymer PHB/PHV. The results of one such experiment are shown in Fig. 3. Following growth on metha-

nol, a mixture of methanol and valeric acid was added to the culture, resulting in the production of the co-polymer PHB/PHV. At the end of the experiment, PHV constituted about 20% of the co-polymer.

Discussion

It is well accepted that PHB and related co-polymers will be used as biodegradable plastics on a large-scale only if their cost of production can be lowered to a value approaching that for non-biodegradable plastics. The substrate represents a major fraction of the manufacturing costs of PHB and is therefore a prime target for potential cost reduction. Methanol, easily obtained from natural gas, is considered one of the most promising substrates for PHB production (Byrom 1987; Collins 1987; Suzuki et al. 1986a, b) although PHB yields (PHB/substrate) will be significantly lower than with some other substrates. Methanol offers several advantages as a fermentation substrate besides being a non-food substrate: low and relatively stable cost, high purity, high availability, easy to transport and to store, high solubility in water and low viscosity.

Although the possibility of producing PHB from methanol is common knowledge, it is surprising to note that few methylotrophic bacteria have been extensively investigated so far, except for the excellent work of Suzuki et al. (1986a–c, 1988) and that of Powell et al. (1980). Some more methylotrophic bacteria have been studied but the work is veiled by "industrial secrecy". It was therefore important to attempt to isolate potentially new PHB-producing methylotrophic bacteria in order to add to the general knowledge about this group of microorganisms and possibly select microorganisms with superior characteristics.

The culture isolation and selection procedures used in this study allowed for the selection of a new isolate of *M. extorquens* initially containing about 25% (by weight) of PHB under the conditions originally used. The isolate certainly belongs to the well-known group of the pink-pigmented facultative methylotrophic bacteria, also known as the PPFMs (Bousfield and Green 1985; Anthony 1982; Green and Bousfield 1983). It is possible that our culture is similar to the culture of *Pseudomonas* sp. K used by Suzuki et al. (1986a) and later identified as *Protomonas extorquens* sp. K (Suzuki et al. 1986b); this remark is based on the fact that the classification of methylotrophic bacteria is sometimes unclear and that Bousfield and Green (1985) have recommended that bacteria of the genus *Protomonas* be classified in the genus *Methylobacterium*. It is interesting to note that soil samples B and C (Table 1), originating from sites contaminated by used oil products for many years, were especially rich in pink-pigmented microorganisms. Some were also found in soil sample F (rich garden soil). Other known methylotrophic PHB-producing bacteria include *M. rhodesianum* Z (Hilger et al. 1991), *Methylosinus trichosporium* (Weaver et al. 1975), *M. organophilum* (Powell et al. 1980), and *Methylococcus parvus* (Asenjo and Suk 1986).

Using methanol as substrate has two characteristics: potential toxicity and volatility. Tests done with our *Methylobacterium extorquens* isolate showed that methanol concentrations above 1.7 g/l began to be inhibitory (Fig. 1) with inhibition becoming significant at concentrations above 5 g/l. Regarding methanol volatility, our studies indicated that this was a negligible problem as volatility is minimized when low concentrations of methanol are used or maintained in cultures. The toxicity and volatility of methanol emphasize the need for accurate on-line measurement and control of methanol concentration, especially at the fermentor level. Attempts to control the addition and concentration of methanol using an open-loop configuration, employing a pump and a programmable timer, gave acceptable results but better results would be expected if a closed loop configuration, requiring on-line measurement of the methanol concentration, were used. A closed loop configuration is preferable as it will be able to respond to culture variations. Such a configuration was used with great success by Suzuki et al. (1986a) to develop a very high cell density process for PHB production.

In this study, no special effort was made to favour PHB accumulation as the emphasis was on partially characterizing the new isolate. Yields of PHB, on a dry weight basis, averaged 25–30% (Fig. 2). This is comparable to yields reported earlier for a few other methylotrophic bacteria (Weaver et al. 1975; Powell et al. 1980; Hilger et al. 1991). Further process development should greatly increase PHB yields in our *M. extorquens* isolate to 60–70% of dry weight, as reported for a few other methylotrophic bacteria after process optimization (Suzuki et al. 1986a; Asenjo and Suk 1986). Our results show also that the *M. extorquens* isolate was able to produce the co-polymer PHB/PHV after addition of the mixture methanol + valerate to the culture (Fig. 3): the PHV-to-PHB ratio was 0.2 at the end of the fermentation. It is well recognized in the field that the co-polymer PHB/PHV has more industrial appeal than the homopolymer PHB. Very little information is available on the production of co-polymers related to PHB by methylotrophic bacteria except for short reports by Dawes (1988) and Haywood et al. (1989). The possible production of other PHB-related co-polymers by *M. extorquens* will be investigated in the near future.

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References

Akita S, Einaga Y, Miyaki Y, Fujita H (1976) Solution properties of poly(D-β-hydroxybutyrate). 1. Biosynthesis and characterization. Macromolecules 9:774–780

American Type Culture Collection (1985) Gherna R, Nierman W, Pienta P (eds) Catalogue of bacteria, phages and rDNA vectors, 16th edn. ATCC, Rockville, Md.

Anthony C (1982) The biochemistry of methylotrophs, chapter 1. Academic Press, New York

Asenjo JA, Suk JS (1986) Microbial conversion of methane into poly-β-hydroxybutyrate (PHB): growth and intracellular product accumulation in a type II methanotroph. J Ferment Technol 64:271–278

Berger E, Ramsay BA, Ramsay JA, Chavarie C (1989) PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol Technol 3:227–232

Bousfield IJ, Green PN (1985) Reclassification of bacteria of the genus *Protomonas* Urakami and Komagata 1984 in the genus *Methylobacterium* (Patt, Cole, and Hanson) Emend. Green and Bousfield 1983. Int J Syst Bacteriol 35:209

Braunegg G, Sonnleitner B, Lafferty RM (1978) A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol 6:29–37

Byrom D (1987) Polymer synthesis by microorganisms: technology and economics. Tibtech 5:246–250

Collins SH (1987) Choice of substrate in polyhydroxybutyrate synthesis. In: Stowell JD, Beardsmore AJ, Keevil CW, Woodward JR (eds) Carbon substrates in biotechnology (Special Publications of the Society for General Microbiology). IRL Press, Oxford, pp 161–168

Dawes EA (1988) Polyhydroxybutyrate: an intriguing biopolymer. Biosci Rep 8:537–547

Dijkhuizen L, Hansen TA, Harder W (1985) Methanol, a potential feedstock for biotechnological process. Tibtech 3:262–267

Green PN, Bousfield IJ (1983) Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov. corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. Int J Syst Bacteriol 33:875–877

Haywood GW, Anderson AJ, Dawes EA (1989) A survey of the accumulation of novel polyhydroxyalkanoates by bacteria. Biotechnol Lett 11:471–476

Hilger U, Sattler K, Littkowsky U (1991) Studies on the growth-associated accumulation of poly-β-hydroxybutyric acid with *Methylobacterium rhodesianum* Z. Zentralbl Mikrobiol 146:83–88

Lafferty RM, Korsatko B, Korsatko W (1988) Microbial production of poly-β-hydroxybutyric acid. In: Rehm HJ, Reed G (eds) Biotechnology, vol 6B. VCH, Weinheim

Large PJ, Bamforth CW (1988) Methylotrophy and biotechnology. Longman Scientific & Technical, Burnt Mill, Harlow, UK

Large PJ, Quayle JR (1963) Microbial growth on C_1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1. Biochem J 87:386–396

Luong JHT (1987) Generalization of Monod kinetics for analysis of growth data with substrate inhibition. Biotechnol Bioeng 29:242–248

Marchessault RH, Okamura K, Su CJ (1970) Physical properties of poly (β-hydroxybutyrate). II. Conformational aspects in solution. Macromolecules 3:735–740

Powell KA, Collinson BA, Richardson KR (1980) Microbiological process for the production of poly (beta-hydroxybutyric acid) and micro-organisms for use therein. European patent application no. 80300432.4

Suzuki T, Yamane T, Shimizu S (1986a) Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph. Appl Microbiol Biotechnol 23:322–329

Suzuki T, Yamane T, Shimizu S (1986b) Kinetics and effect of nitrogen source feeding on production of poly-β-hydroxybutyric acid by fed-batch culture. Appl Microbiol Biotechnol 24:366–369

Suzuki T, Yamane T, Shimizu S (1986c) Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl Microbiol Biotechnol 24:370–374

Suzuki T, Deguchi H, Yamane T, Shimizu S, Gekko K (1988) Control of molecular weight of poly-β-hydroxybutyric acid produced in fed-batch culture of *Protomonas extorquens*. Appl Microbiol Biotechnol 27:487–491

Tani Y (1984) Microbiology and biochemistry of methylotrophic yeasts. In: Hou CT (ed) Methylotrophs: microbiology, biochemistry, and genetics, CRC Press, Boca Raton, Fla.

Toennies G, Gallant D (1949) The relation between photometric turbidity and bacterial concentration (bacterimetric studies IV). Growth 13:7–20

Weaver TL, Patrick MA, Dugan PR (1975) Whole-cell and membrane lipids of the methylotrophic bacterium *Methylosinus trichosporium*. J Bacteriol 124:602–606