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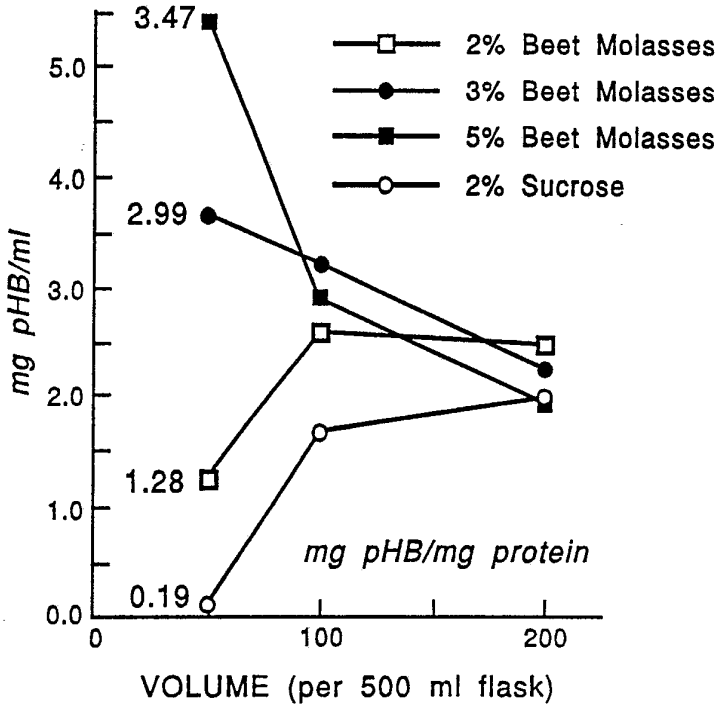

(57) Abstract

A mutant strain of *Azotobacter vinelandii* exhibits hyperproduction of poly-hydroxyalkanoates during its growth. Poly-hydroxyalkanoates yield is further enhanced when sugar beet molasses is used as carbon source.

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HYPERPRODUCTION OF POLY-HYDROXYALKANOATES DURING EXPONENTIAL GROWTH BY MUTANT STRAINS OF AZOTOBACTER VINELANDII

TECHNICAL FIELD

This application is based upon a continuation-in-part of U.S. Application No. 268,789 filed on November 9, 1988. This invention relates to the hyperproduction of certain poly-hydroxyalkanoates during growth of mutant strains of Azotobacter vinelandii.

BACKGROUND ART

Poly-hydroxyalkanoates may be characterized as polymers of one or more monomers residues of the formula

$$-OCR_1R_2(CR_3R_4)_n-CO \qquad I$$

wherein n is an integer from 1 to 5; and R_1, R_2, R_3 and R_4 are independently selected from the group consisting of a hydrogen atom and C_{1-5} alkyl radicals. The polymers include poly-ß-hydroxybutyrate (PHB) and copolymers of 3-hydroxybutyrate and 3- or 4-hydroxyvalerate (the PHBV's).

Poly-ß-hydroxybutyrate (PHB) is a biodegradable, biocompatible, thermoplastic made by microorganisms [Baptist, J.N., 1962, U.S. Patent 3,036,959]. In the cell, PHB is an intracellular storage material synthesized and accumulated during unbalanced growth. It accumulates as distinct white granules that are clearly visible in the cytoplasm of the cell. Under conditions of nutrient starvation, PHB is used by the cell as an internal reserve of carbon and energy. Many bacteria including those in the soil, are capable of PHB production and breakdown. Animal cells do not form PHB but are able to break down the polymer.

PHB is a homopolymer of repeating 3-hydroxybutyric acid units. Copolymers with hydroxyvaleric acid (PHBV) can be made by "precursoring" (e.g.) adding propionic acid to the culture during growth [Holmes et al., 1981, European Patent 0,052,459 and 1984 U.S. Patent 4,477,654]. This modification to the PHB homopolymer reduces the crystallinity and melting point of the plastic, allowing film formation and melt-extrusion applications. PHB plastics also are used in microelectronics applications exploiting the piezoelectric properties of PHB. An immediate market for PHB plastics will be in high value added products (e.g.) biodegradable surgical pins, plates, pegs and

sutures, implants for drug delivery, and possibly meshes can be used as artificial skin materials. PHB/PHBV derived plastics also have considerable potential application as biodegradable bulk plastics, replacing non-biodegradable products formed from polypropylene or polyethylene. Development of many of these products is ongoing in view of their potential uses.

To date, production of PHB/PHBV in the cell occurs during imbalanced growth. Usually this is the stationary phase of bacterial growth, but this can be induced in an actively growing culture by imposing a nutrient (O_2, nitrogen, phosphate, or sulfate) limitation in the presence of excess carbon source. during this imbalance, NADH accumulates and exerts a feedback repression on various enzymes whose activities are essential for the continued growth of the cell. NADH can be oxidized to NAD^+, eliminating this growth inhibition, by the action of acetoacetyl CoA reductase and the polymerization of acetoacetyl CoA into PHB.

NAD^+ is nicotinamide adenine dinucleotide and NADH is its reduced form. NAD^+ is a major electron acceptor in the oxidation of fuel molecules in the cell. NAD^+ fulfills this function by accepting two electrons and two hydrogen ions from substances its oxidizes. Thus, NAD^+ becomes NADH. Stryer, Biochemistry 2d ed., W.H. Freeman and Company, San Francisco, pp. 244-246.

Azotobacter vinelandii is a harmless soil microbe that has an obligate O_2 requirement for growth and can use N_2 as a nitrogen source via nitrogen fixation. A. vinelandii normally produces PHB by the methods noted above and much of the early work concerning PHB synthesis was conducted in Azotobacter species. To the best of applicants knowledge the production of PHBV from A. vinelandii has never been reported. Azotobacter species that produce large amounts of PHB have been reported, but these cells have been unstable and also produce large amounts of capsule and slime, which interfere with PHB extraction and decrease the efficiency of conversion of carbon substrate to PHB.

At present, PHB/PHBV is produced commercially by ICI in the U.K., using a strain of Alcaligenes eutrophus growing in a glucose salts medium. Their fermentation involves a rapid growth phase (60h), followed by phosphate-limitation and glucose

feeding (an additional 48-60h). During phosphate-limited growth, PHB is formed and may account for 75% of the total cell weight. The yield per litre is dependent on the initial cell mass and theoretical yields of 0.33 t PHB t^{-1} glucose have been calculated [Byrom, D., Trends in Biotechnology 5: 246-250, 1987].

Presently, PHB/PHBV production involves a long fermentation time, in the stationary phase of growth, to obtain high levels of PHB. Different nutrient limitations have been imposed during stationary phase to enhance PHB production.

Currently, PHB/PHBV production is limited by a relatively long fermentation time, dependence on amount of PHB/PHBV produced upon continued cell activity after the active (exponential) phase, dependence on amount of PHB produced upon a pregrowth period to achieve an initial cell mass such that a certain amount of the carbon source is used to produce cell mass rather than PHB/PHBV, and the need to use relatively expensive substrates (such as glucose) for fermentation.

To date, there appears to have been an effort to increase PHB/PHBV yield in industrial applications by increasing batch size. Because PHB/PHBV is considered a secondary metabolite, produced in the stationary phase of growth after active cell growth, the possibility of increasing yield by bringing about PHB/PHBV production during exponential growth has not been addressed and successfully exploited.

Likewise, the possibility of exploiting relatively unrefined carbon sources in PHB/PHBV production has not been successfully exploited. The unrefined carbon sources are typically more complex, less refined, or sometimes unpurified materials or even mixtures of materials, which are not necessarily or pure or defined composition. Examples of unrefined carbon sources include blackstrap molasses (sugar cane molasses), sugar-beet molasses, malt extract, maltose, corn syrup, carbohydrates and phenols in industrial or municipal wastes. Instead of said unrefined substances, relatively pure carbon sources of defined composition, such as glucose have been used for industrial PHB/PHBV production to date.

DISCLOSURE OF THE INVENTION

According to an aspect of the invention, a genetically transformed biologically pure microorganism of the species Azotobacter vinelandii has the identifying characteristics of ATCC 53799.

According to another aspect of the invention, a biologically pure culture of a genetically transformed microorganism of the species Azotobacter vinelandii, the genetically transformed microorganism having the identifying characteristics of ATCC 53799, when cultured is capable of hyperproducing a polymer of one or more monomers residues of the formula

$$-OCR_1R_2(CR_3R_4)_n-CO \qquad I$$

wherein n is 1 or 2; and R_1, R_2, R_3 and R_4 are independently selected from the group consisting of a hydrogen atom and C_{1-2} alkyl radicals in a recoverable quantity upon fermentation in an aqueous nutrient medium containing assimilable nutrients. The hyperproduction of the polymer occurs during the exponential growth of the mutant microorganism.

According to another aspect of the invention, a genetically transformed bacterium of the species Azotobacter vinelandii is provided, the genetically transformed bacterium having the identifying characteristics of:

(i) extremely abundant PHB granules in cells;

(ii) dense white colonies of cells;

(iii) very turbid culture which reaches an $O.D._{620}$ of 10 and looks like cow's milk after 24 hours of growth;

(iv) no formation of substantial capsule or slime; and

(v) hyperproduction of PHB during the exponential growth of the mutant microorganism.

According to another aspect of the invention, there is provided a process for producing a polymer of one or more monomers residues of the formula

$$-OCR_1R_2(CR_3R_4)_n-CO \qquad I$$

wherein n is 1 or 2; and R_1, R_2, R_3 and R_4 are independently selected from the group consisting of a hydrogen atom and C_{1-2} alkyl radicals comprising:

(i) culturing genetically transformed bacteria of the species Azotobacter vinelandii, the genetic

transformants having the identifying characteristics of ATCC 53799 in a culture medium having a source of assimilable nutrients; and

(ii) recovering polymer from the bacteria.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a plot comparing Azotobacter vinelandii strain UW ATCC 13705(•) and Azotobacter vinelandii strain UWD ATCC 53799(○) with respect to increase in turbidity (plot A) and protein content (plot B) during incubation at 30°C with vigorous aeration.

Figure 2 is a plot comparing Azotobacter vinelandii strain UW ATCC 13705(•) and Azotobacter vinelandii strain UWD ATCC 53799(○) with respect to production of PHB (plot A) and consumption of glucose (plot B) during growth. The information depicted in Figure 2 is derived from analyses performed on the cells and culture fluids from Figure 1.

Figure 3 is a plot comparing effect of varying degrees of aeration and effect of different carbon sources on PHB production by the Azotobacter vinelandii strain UWD ATCC 53799.

BEST MODE FOR CARRYING OUT THE INVENTION

The poly-hydroxyalkanoates of the present invention may be co or homopolymers. Preferably in formula 1, n is 1 or 2 and R_1, R_2, R_3 and R_4 are independently selected from the group consisting of a hydrogen atom, a methyl radical and an ethyl radical. If the polymer is a homopolymer and n is 1 and one of R_1 and R_2 is a methyl radical and the other is a hydrogen atom and both R_3 and R_4 are hydrogen atoms, the polymer is poly-ß-hydroxybutyrate (PHB) . If the polymer is a copolymer and n, R_1, R_2, R_3 and R_4 are as described immediately above in one monomer residue and in the other monomer residue, n is 2, one or R_1 and R_2 is a methyl radical and the other is hydrogen and R_3 and R_4 (both occurrences) are hydrogen the polymer is PHBV. Preferably in the above copolymer the mole ratio of the butyrate residues to the valerate residues is from about 70:30 to about 99:1, most preferably above 95:5.

If it is desired to produce a copolymer in accordance with the present invention it is necessary to add a precursor. The precursor may be a C_{5-9} odd numbered alkanoic acid or a salt there of such an alkali metal salt such as sodium or potassium

salts. Preferably the precursor is sodium valerate or valeric acid. The precursor may be present in the growth medium in a concentration from 5 to 45, preferably 10 millimolar ("mM"). The precursor should be added during the time when the cells are actively forming polymer. The percent comonomer (e.g. valerate) in the polymer may be increased by adding higher concentrations of precursor, or by repeated addition of precursor, or by addition of the precursor shortly (between 4 to 8 hours) before harvesting.

The bacterial transformant, according to this invention, which for the purpose of reference in the detailed description is identified as UWD, was derived from the species Azotobacter vinelandii. It is understood that the invention encompasses not only the particular UWD, but all derivatives thereof and other related microorganisms having similar taxonomic descriptions. The Azotobacter genus is described in Bergey's Manual of Determinative Bacteriology, Vol. 1., pp. 219-229., 1984, N.R. Krieg and J.G. Holt (ed.), Williams and Wilkins, Baltimore, and J.P. Thompson and V.B.D. Skerman, 1979. Azotobacteraceae: The taxonomy and ecology of the aerobic nitrogen-fixing bacteria, Academic Press, New York, pp. 168-69, pp. 178-79.

Azotobacter vinelandii wild-type, which for the purpose of reference in the detailed description is identified as OP, is readily available and has been investigated by various groups. Azotobacter vinelandii OP is deposited at American Type Culture Collection under accession number ATCC 13705. It is understood that throughout the specification, the generally accepted nomenclature "ATCC" for the American Type Culture Collection will be used. ATCC is located at 12301 Parklawn Drive, Rockville, Maryland, U.S.A. All deposits as ATCC are given accession numbers which are referred to throughout the specification. The University of Wisconsin subculture of Azotobacter vinelandii OP is identified as UW for the purpose of reference in the detailed description. The UWD transformant, according to this invention, was developed and isolated by transforming UW cells with DNA prepared from the mutant Azotobacter vinelandii ATCC 63800 (which for the purpose of reference in the detailed description is identified as strain 113) such as defined in the following Example. It is

appreciated that there are many techniques available for inducing such mutation and that there are many techniques available for transforming bacterial cells or otherwise changing their genetic composition and such other techniques are contemplated herein although not specifically exemplified.

The isolated genetic transformant has been characterized in the following Examples and has been deposited at the American Type Culture Collection. The deposit was made on August 10, 1988 under accession number ATCC 53799. The taxonomical characteristics of the UWD strain are as follows.

The UWD strain shares characteristics with strain UW (OP, ATCC 13705) except that it forms excess PHB during exponential growth, it is resistant to rifampicin (20μg/ml), it has a white colony colour, and it forms the fluorescent-green pigment under iron-sufficient conditions.

Basic characteristics: Large ovoid cells, Gram-negative, pleomorphic ranging from rods to cocci. The cells are motile by peritrichous flagella, substantial capsules are not formed and the cells form fragile cysts only after very long incubation (several months to a year). Growth is strictly aerobic and nitrogen is fixed aerobically. Substrate utilization characteristics have been well defined and agree with Bergey's Manual of Determinative Bacteriology, Vol. 1, pp. 219-229, 1984. N.R. Krieg and J.G. Holt (ed.), Williams and Wilkins, Baltimore, and J.P. Thompson and V.B.D. Skerman, 1979. Azobacteraceae: The taxonomy and ecology of the aerobic nitrogen-fixing bacteria, Academic Press, New York, pp. 168-69, pp. 178-179.

The taxomonic description of strain 113 is as follows.

This strain is derived from A. vinelandii ATCC 12837 by NTG mutagenesis. It shares characteristics with ATCC 12837 except it is resistant to rifampicin (20μg/ml) and it forms poly-ß-hydroxybutyrate (PHB) during exponential growth.

Basic characteristics: Large ovoid cells, Gram-negative, pleomorphic ranging from rods to cocci. The cells are motile by peritrichous flagella, form capsules and form cysts in older cultures. Iron-limited cultures produce a fluorescent yellow-green pigment. Growth is strictly aerobic and nitrogen is fixed aerobically. Substrate utilization characteristics have been well defined and agree with Bergey's Manual of Determinative

Bacteriology, Vol. 1, pp. 219-229, 1984, N.R. Krieg and J.G. Holt (ed.), Williams and Wilkins, Baltimore, and J.P. Thompson and V.B.D. Skerman, 1979. Azotobacteraceae: The taxonomy and ecology of the aerobic nitrogen-fixing bacteria, Academic Press, New York, pp. 168-69, pp. 178-79.

The UWD transformant was developed and isolated in accordance with the following preferred method. Cells of strain UW were obtained from the Department of bacteriology at University of Wisconsin. This strain of cells is a capsule-negative wild-type, which were genetically transformed with DNA prepared from cells of stain 113 Azotobacter vinelandii 53800, which is a rifampin-resistant strain derived by NTG mutagenesis of the capsule-positive strain ATCC 12837.

Stain 113 ATCC 53800 was produced by exposing Azotobacter vinelandii strain ATCC 12837 to 100 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG) in Burk buffer, pH 7.2, for 30 min. Survivors were plated on Burk medium containing 1% glucose, 1.8% agar and 20 μg rifampin/ml. Strain 113 was, therefore, selected as a nitrogen-fixing, rifampin-resistant strain of ATCC 12837. The hyperproduction of PHB by strain 113 was an unselected mutation that also has occurred during the NTG mutagenesis procedure.

DNA for transformation was prepared as a crude lystate material. A thick suspension of strain 113 was prepared in 15 mM saline-15 mM sodium citrate buffer, pH 7.0, containing 0.05% sodium dodecyl sulfate. this suspension was heated at 60°C for 60 min. in a waterbath. When cool, this lystate containing crude (unpurified) DNA was used directly in transformation assays. Optimal conditions for generation of competent strain UW (or Azotobacter vinelandii in general) which can take up this crude DNA, are documented in Page and von Tigerstrom, 1978. Can. J. Microbial. 24:1590-1594. Optimal conditions for the transformation of these competent cells by the crude DNA are documented in Page and Von Tigerstrom, 1979. J. Bacteriol. 139:1058-1061.

The preferred procedure used to transform UW cells with strain 113 DNA is described in Page, W.J. 1985. Can. J. Microbial. 31:659-662. While almost any strain of Azotobacter vinelandii can be transformed by strain 113 DNA, the most

successful transformations for the purpose of producing maximal amounts of readily extractable PHB are the transformations of capsule-negative strains. Of these, Azotobacter vinelandii strain OP ATCC 13705 is a readily available strain held in cultural collections. The strain that was transformed to create the UWD transformant was strain UW, the University of Wisconsin copy of strain OP.

Transformation of stain UW with strain 113 DNA results in a rifampin-resistance transformation frequency of about 1.0×10^{-4} to 8.7×10^{-3} per viable cell plated (i.e. at best 8.7 transformants per 1000 cells plated). This is a readily reproducible frequency of transformation. Of these rifampin-resistant colonies, about 13% appeared to have streaks of white or white sectors within the normal pinkish-tan cell mass. When these sectoring colonies were restreaked, they gave rise to sectoring colonies and dense white colonies at a ratio of about 1:1. the dense white colonies were then selected and designated UWD. This procedure and these results are reproducible and will readily generate UWD cells of this invention.

UWD is readily separated from the UW stock because UWD is resistant to rifampin and UW is not resistant.

Therefore, on plates containing solid medium and 20 μg rifampin/ml, only colonies of UWD (or other rifampin resistant cells) will grow.

The separation of PHB hyperproducing cells from the general population of transformed cells is readily replaceable, because the sectoring colony phenotype is quite distinctive and upon subculture it readily generates solid white colonies. Non-hyperproducing cells result in pinkish-tan colonies under the same conditions.

Quite surprisingly it was discovered that a certain number of so transformed cells produced dense-white colonies, rather than the normal translucent tan wild-type colonies of the UW strain. The cells from these dense colonies of the UW strain. The cells from these dense colonies (the UWD cells) were packed with PHB granules, while the wild-type (strain UW) contained only a few small granules of PHB.

It is believed that in theory the production of PHB/PHBV in the mutant is due to a genetic defect in strain 113 and the UWD

cells concerning the NADH oxidase that normally recycles NAD^+ via respiratory oxidation of NADH. As a result of this defect, the cell accumulates NADH and must turn-on PHB/PHBV synthesis in order to grow. Therefore, PHB/PHBV are formed during active (balanced; exponential) growth, the exact opposite of the normal conditions promoting PHB/PHBV formation in the wild-type. Although this theory appears to be borne out by the examples, it is understood that the principles of the invention should not be limited to this theory.

Because PHB/PHBV type polymers are formed during exponential growth, conditions which enhance growth also increase PHB/PHBV type polymer formation. For example, vigorous aeration (rather than O_2-limitation) promotes faster use of glucose and faster production of PHB/PHBV type polymers. Nitrogen-fixing UWD cells also produce PHB.

Strain 113 also produces large amounts of PHB, but also produces large amounts of capsule and slime. The UWD cells of this invention do not form substantial capsule and slime and therefore only convert the bulk of the sugar into cell mass (like strain UW) and PHB.

Solid media for the maintenance of cultures contained 1.5 to 1.8% (w/v) agar.

Production of PHB was demonstrated in batch cultures. The volumes of culture were in the range 20-50% culture volume: flask or vessel volume with rotary shaking (to increase aeration and mixing) at 175 to 300 rpm (normally 250 rpm was used). Incubation temperatures were found to be 30-35°C for optimal yields.

The nitrogen source used in the cell culture is preferably N_2 (from air) or ammonium acetate at 1.1 to 2.2 g/L.

Various carbon sources were added to the culture medium at 1 to 5% (w/v) concentration. PHB production was best with reduced hexose, mono and disaccharide carbon sources (glucose, sucrose, maltose) or with sodium gluconate or glycerol, and was much lower with more oxidized or short-chain carbon sources (acetate, ethanol). Glucose and sucrose are relatively expensive refined substrates for PHB production. Sucrose does not have to be "inverted" before use by the UWD cells but fructose is poorly used for PHB production. Because PHB

formation is not dependent on nutrient limitation, cheaper unrefined carbon sources can be used by the UWD cells. Good production of PHB has been obtained using 2% (w/v) corn syrup, sugar-beet molasses, blackstrap and cooking molasses, and more refined grades of refiner's molasses. The UWD cells grow well in media containing at least 5% (w/v) molasses or corn syrup, however, the yield of PHB ml^{-1} is usually not greater than that obtained with 2 or 3% molasses or corn syrup except for a further preferred embodiment of this invention as noted below. On these impure carbon sources the wild-type strains did not produce detectable or significant amounts (<0.1 mg/ml) of PHB.

Growth of UWD cells and yield of PHB under intense aeration conditions (culture volume 10% of vessel volume) were unexpectedly increased when sugar beet molasses was used as the carbon source. Under intense aeration conditions (as established by the culture volume being 10% of the vessel volume during shaker flask culture), a yield of 5.42 PHB/ml was obtained with 5% sugar beet molasses, a 42-fold increase over the yield obtained with 2% sucrose as carbon source under the same conditions. Sugar beet molasses concentrations greater than 5%, or increases in sucrose only, had no further beneficial effect. This growth promotional effect of sugar beet molasses under intense aeration conditions was observed with all ten different samples of sugar beet molasses examined.

Increasing PHB yields by use of an unrefined carbon source such as sugar beet molasses offers a significant reduction in production costs.

Various aspects of the invention are demonstrated in the following examples:

Example 1

PHB was formed and extracted from cultures of the UWD transformant as follows. The growth medium in which UWD cells were cultured to yield PHB was a minimal salts medium composed of g/L: KH_2PO_4, 0.2; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.2; $CaSO_4.2H_2O$, 0.1: $FeSO_4.7H_2O$, 0.005; $Na_2MoO_4.2H_2O$, 0.00025; at a pH 7.2.

When UWD cells were grown in the described minimal salts medium initially containing 10 mg ml^{-1} $C_6H_{12}O_6$, the medium became very turbid and by 24 h reached an $O.D._{620}$ of 10 and looked like cow's mile as shown in Figure 1. This Figure is a comparison of

the UW ATCC 13705 strain (•) and the UWD ATCC 53799 strain (○) for increase in turbidity (A) and protein content (B) during incubation at 30°C with vigorous aeration culture volume 20% of vessel volume. In the same time period, strain UW attained an $O.D._{620}$ of 3 and produced a tan coloured suspension as shown in Figure 1.

Analysis of the growth of the cultures showed that the UWD cells (a) formed PHB as soon as they started using glucose, (b) formed PHB during exponential growth, (c) used the glucose in the medium faster than strain UW, (d) produced 2 mg PHB ml^{-1} in 24h, compared to 0.25 mg PHB ml^{-1} produced by strain UW under the same conditions as shown in Figure 2.

In the analysis, PHB was isolated by the method of Law and Slepecy, 1961. J. Bacterial 82: 33-36. Cells containing PHB were collected by centrifugation and dissolved in bleach for 1h at 40°C. The residue was washed with distilled water, 95% ethanol and acetone. The dry weight of the residue was then determined. The residue was 100% soluble in chloroform, which solubility is characteristic of PHB. Selected samples were also subjected to the colorimetric assay described by Law and Slepecky to further confirm that the residue was PHB. The dry weight of the cells before extraction was determined after concentration by centrifugation and washing twice with distilled water to remove media contaminants.

Example 2

The effect of different carbon sources of PHB production by the UWD strain was investigated. Various carbon sources were evaluated where the selected source of nitrogen was either ammonia or N_2. The results of the investigation are itemized in the following Table 1.

Table 1

Medium[c]	Ammonium-grow[a] PHB mg ml^{-1}	Nitrogen-fixing[b] PHB mg ml^{-1}
1% Glucose	1.88	1.86
30 mM Acetate[d]	0.09	0.08
1% Glucose + 30 mM acetate[d]	2.05	1.27
1% Maltose	2.08	1.09
2% Sucrose	0.96	1.44
1% Ethanol	0.17	0.03
1% Glycerol	1.27	0.11

[a]All cultures contained 15 mM ammonium acetate.

[b]All cultures contained N_2 as sole-N source.

[c]Cultures were grown with vigorous aeration at 30.C for 24h (culture volume 20% of vessel volume).

[d]Total acetate concentration.

Example 3

The investigations of Example 2 were extended to consider the impact of less expensive carbon sources on PHB. The results of this investigation are summarized in the following Table 2.

Table 2

Carbon Source[a]	PHB mg ml^{-1}	%PHB [cell dry weight]$^{-1}$
1% Glucose	2.48	60
1% Sucrose	2.39	68
1% Fructose	0.64	53
2% Corn Syrup	2.38	57
2% Black Strap Molasses	2.33	54
2% Cooking Molasses	2.49	54
2% Refiner's Molasses	2.40	56
2% Sugar-Beet Molasses	2.58	60

[a]Salt medium plus 15 mM ammonium acetate, incubated at 30°C with vigorous aeration for 24h (culture volume 20% of

vessel volume). Molasses media were adjusted to pH 7.0 with NaOH.

Example 4

Under intense aeration conditions (culture volume 10% of vessel volume) PHB production was less than 1 mg PHB/ml, as seen in Figure 3. PHB production at this level of aeration was stimulated by sugar beet molasses, as set out in Figure 3 and Table 3.

UWD cells were grown in a salts medium as in Example 1 and PHB was isolated and determined as described in Example 1. cell protein was determined colorimetrically (Page & Huyer (1984) J. Bacteriol., v. 158, pp. 496-502). Beet molasses samples, which contained sucrose as the predominant sugar, were obtained from both Canada and the U.S.A.

The aeration rate was varied by changing the culture volume per 500 ml. Erlenmeyer flask, with the most intense aeration at 50 ml culture volume per flask. All cultures received a 2% v/v inoculum and were incubated at 28-30°C with shaking to perform aeration of the culture at 225-250 rpm for 24h.

Table 3

Sucrose %	% Beet Molasses	PHB Yield mg/ml	PHB % dry wt
0	1	0.04	3
0	2	1.12	32
0	3	3.40	54
2	0	0.28	15
2	0.5	0.81	29
2	1	1.51	38
2	2	4.33	61
2	3	5.65	66

Strain UWD formed only a minimal amount of PHB when grown in 2% w/v sucrose under intense aeration conditions (Figure 3). However, strain UWD had higher yields PHB/ml and PHB/protein when grown with 2% or 3% beet molasses in the intensely aerated culture (Figure 3). The yield increased to 5.42 mg PHB/ml with 5% beet molasses, a 42-fold increase over that obtained in the sucrose control culture. Although the culture protein content

increased 2.2-fold in the 5% beet molasses culture over the control, the PHB protein increased 18-fold, which indicated a disproportionated channelling of carbon into PHB at intense aeration. This channelling was not apparent at lower aeration, where the PHB/protein yield remained constant for all samples including the control. A mean yield of 6.77 ± 0.5 mg PHB/ml (3.27 ± 0.7 mg PHB/mg protein) was obtained with 10 different sugar beet molasses samples used at 5% w/v in salts medium.

The addition of just 0.5% beet molasses to the sucrose control increased the yield of PHB in these cultures (Table 3). These data suggest that the growth promotional substances in sugar beet molasses are relatively abundant. Higher yields of PHB also were obtained with 1-3% molasses if additional sucrose was added (Table 3).

Example 5

In a similar manner A. vinelandii (ATCC 53799) was cultured in 5% beet molasses in minimal salts medium, essentially as above including incubation, except that sodium valerate was added to a final concentration of 10 mM at the times indicated in Table 4. The cells were harvested and extracted and the percent of polymer (PHBV) and was recorded and the percent of valerate residue in the polymer analysed. The results are set forth in Table 4.

Table 4

Time of Valerate Addition (h)	PHBV @ 24h			% Valerate in Polymer		
	mg/ml	%	mg/mg protein	16h	20h	24h
No addn.	7.04	65.7	4.09	ND	ND	0
0	5.90	60.4	3.07	ND	ND	0
4	4.07	54.9	2.42	ND	ND	1.6
8	5.03	57.7	2.86	16.6	ND	8.5
12	6.49	73.2	3.45	19.1	21.3	14.5
16	6.81	70.7	4.15	ND	ND	8.2

These results show that PHV contents between 1.6 to 21.3% can be generated by a single addition of valerate. The timing of addition is important to coincide with active PHB formation (8 to 12 hour period after innoculation, in this case). It

seems to take some time for the valerate to taken into the cell and appear in the polymer (approximately 4 to 8 hours).

Example 6

In the same manner as Example 5, A. vinelandii (ATCC 53799) was cultured except that sodium valerate was added to a final concentration of 10 mM at each time of addition. The harvest and analysis were as in Example 4. The results are set forth in Table 5.

Table 5

Time of Valerate Addition (h)	PHBV @ 24h			% Valerate in Polymer		
	mg/ml	%	mg/mg protein	16h	20h	24h
12	4.29	60.0	3.25	ND	ND	19.7
12, 16	4.15	56.5	3.24	ND	14.6	23.4
12, 14, 16	4.04	57.6	3.37	9.2	13.8	27.1

Repeat addition of precursor tend to give higher final concentration of comonomer. The yield was about 30% comonomer.

It is appreciated that there are a variety of techniques available to measure extent or degree of aeration in a microbial culture system. It is also appreciated that in scaling up culture conditions from laboratory culture conditions to industrial culture conditions, there is usually an adjustment to the extent of aeration to compensate for the considerably increased culture volume and the varying types of industrial types of culture aerating equipment. However, it is generally understood that a striking culture process parameter noted in the laboratory reasonably predicts that the same striking process parameter will hold true in an industrial scale system.

The results of Example 4 clearly indicate the striking process parameter that under intense aeration conditions where the carbon source of the nutrients is sugar beet molasses, the hyperproduction of PHB can be increased up to some 42-fold compared to sucrose as the carbon source under the same conditions. By use of the term "intense aeration" it is understood to mean a type of culture aeration which in commercial production would be equivalent to the type of aeration exhibited in Example 4 and shown in Figure 3. In that example the volume of air in the culture vessel ranges from

approximately 90% down to 60% of vessel volume and the culture volume ranges from approximately 10% up to 40% of the vessel volume (according to the laboratory test). It can therefore be predicted that in a commercial culture system, the use of sugar beet molasses in the range of 2 to 5% w/v and an intense degree of aeration equivalent to the highest degree of aeration will considerably enhance the hyperproduction of PHB by the mutant microorganism of this invention.

INDUSTRIAL APPLICABILITY

Although preferred embodiments of the invention are described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

CLAIMS

1. A process for producing a polymer of one or more monomers residues of the formula

$$-OCR_1R_2(CR_3R_4)_n-CO \qquad I$$

wherein n is 1 or 2; and R_1, R_2, R_3 and R_4 are independently selected from the group consisting of a hydrogen atom and C_{1-2} alkyl radicals comprising:

(i) culturing genetically transformed bacteria of the species Azotobacter vinelandii, the genetic transformants having all the identifying characteristics of ATCC 53799 in a culture medium having a source of assimilable nutrients; and

(ii) recovering polymer from the bacteria.

2. A process to claim 1, wherein the species Azotobacter vinelandii having all the identifying characteristics of ATCC 53799, when cultured has the identifying characteristics:

(i) extremely abundant PHB granules in cells;

(ii) dense white colonies of cells;

(iii) very turbid culture which reaches an $O.D._{620}$ of 10 and looks like cow's milk after 24 hours of growth;

(iv) no formation of substantial capsule or slime; and

(v) hyperproduction of PHB during the exponential growth of the mutant microorganism.

3. A process of claim 2, said source of nutrients being such as to induce exponential growth.

4. A process of claim 3, wherein mutant microorganisms produce said polymer, said source of nutrients including unrefined carbon sources of a type or types suitable to maintain said bacteria and to be used by said bacteria to produce said polymer in a recoverable quantity.

5. A process according to claim 4, wherein said unrefined carbon sources are selected from the group consisting of blackstrap molasses, sugar-beet molasses, malt extract, maltose and corn syrup.

6. A process according to claim 5, wherein said bacteria are cultured at intense aeration, said source of nutrients comprising sugar beet molasses.

7. A process according to claim 6, wherein said sugar beet molasses is at concentration up to about 5% w/v.

8. A process according to claim 7, said source of nutrients further comprising sucrose.

9. A process according to claim 5, wherein said nutrients further include up to 45 mM of one or more odd numbered C_{5-9} alkanoic acids or salts thereof.

10. A process according to claim 8, wherein said polymer is a homopolymer and in formula 1, n is 1, one of R_1 and R_2 is a hydrogen atom and the other is a methyl radial and R_3 and R_4 are hydrogen atoms.

11. A process according to claim 9, wherein in said further nutrients are valeric acid or sodium or potassium valerate.

12. A process according to claims 11, wherein said further nutrient is added from 8 to 12 hours following innoculation.

13. A process according to claim 12, wherein said polymer is a copolymer wherein in formula 1 for the first monomer residue n is 1, one of R_1 and R_2 is a hydrogen atom and the other is a methyl or ethyl radical and R_3 and R_4 are hydrogen atoms and in formula 1 for the second monomer residue n is 2, one of R_1 and R_2 is a hydrogen atom and R_3 and R_4 are hydrogen atoms.

14. A process according to claim 13, wherein the mole ratio of the first monomer residue to the second monomer residue is from 70:30 to 95:5.

15. A process according to claim 14, wherein the mole ratio of the first monomer residue to the second monomer residue is greater than 95:5.

FIG. 1

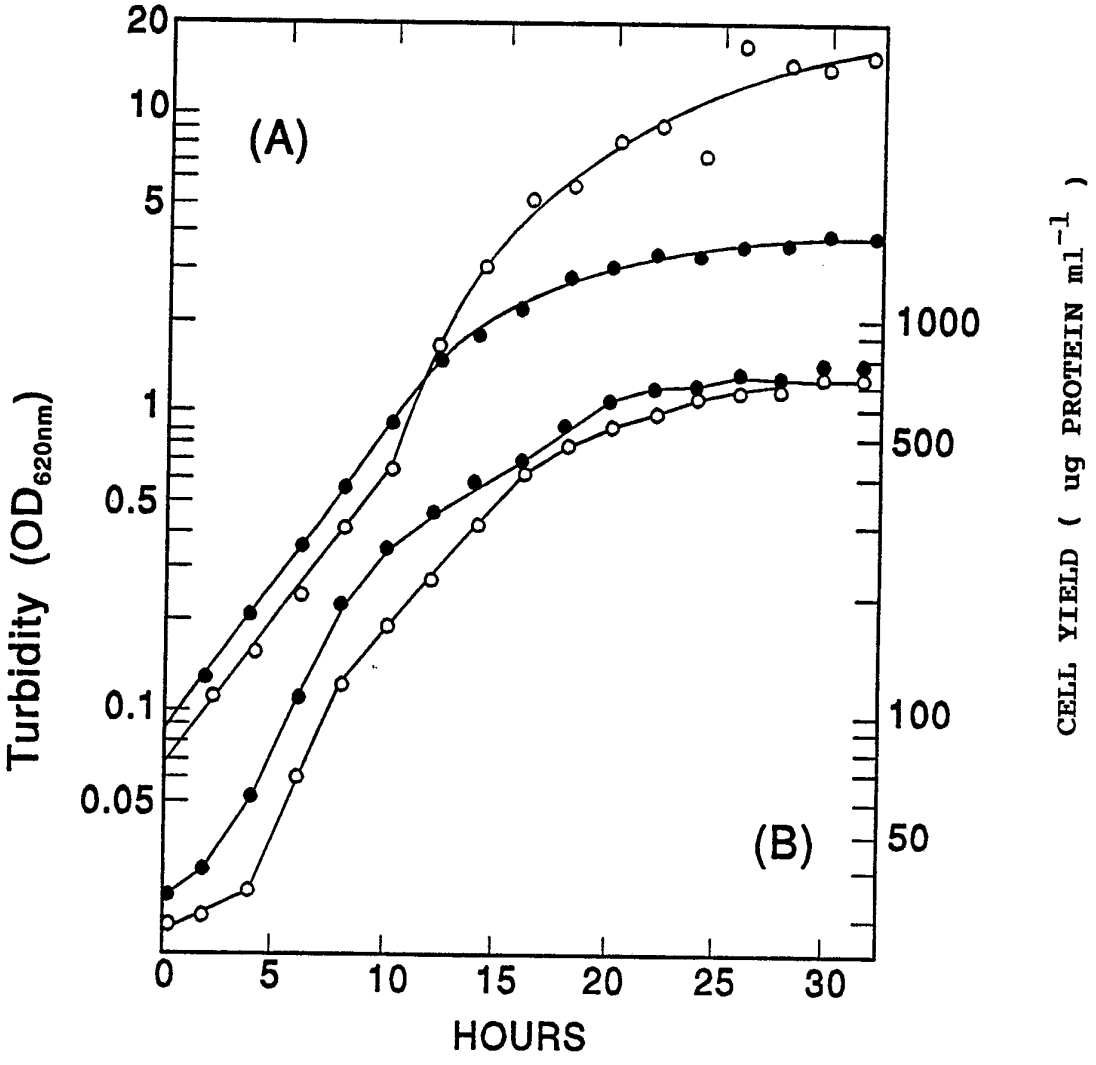

20
10
5
1
0.5
0.1
0.05
(A)
(B)
Turbidity (OD_{620nm})
1000
500
100
50
CELL YIELD (ug PROTEIN ml^{-1})
0
5
10
15
20
25
30
HOURS

FIG. 2

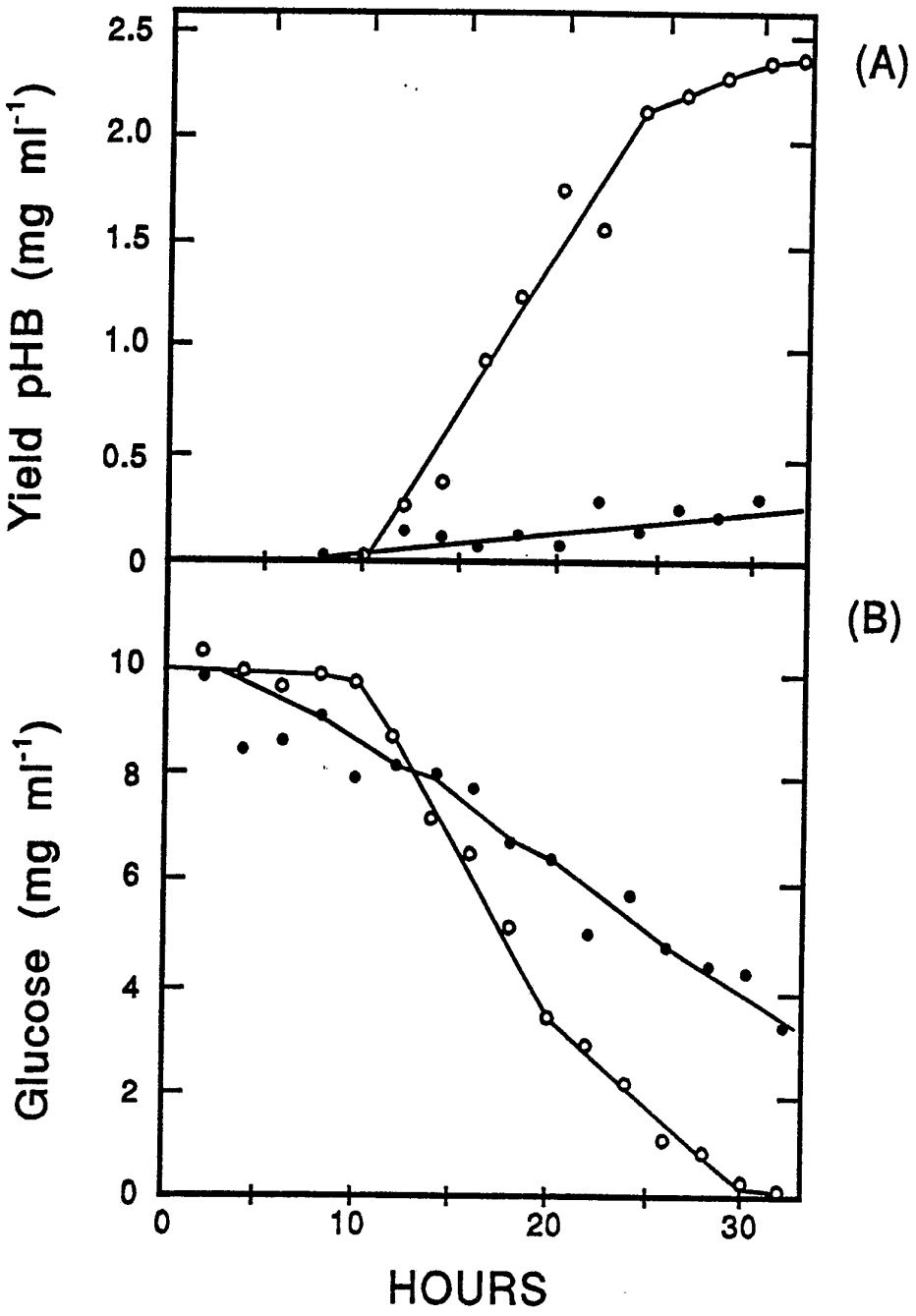
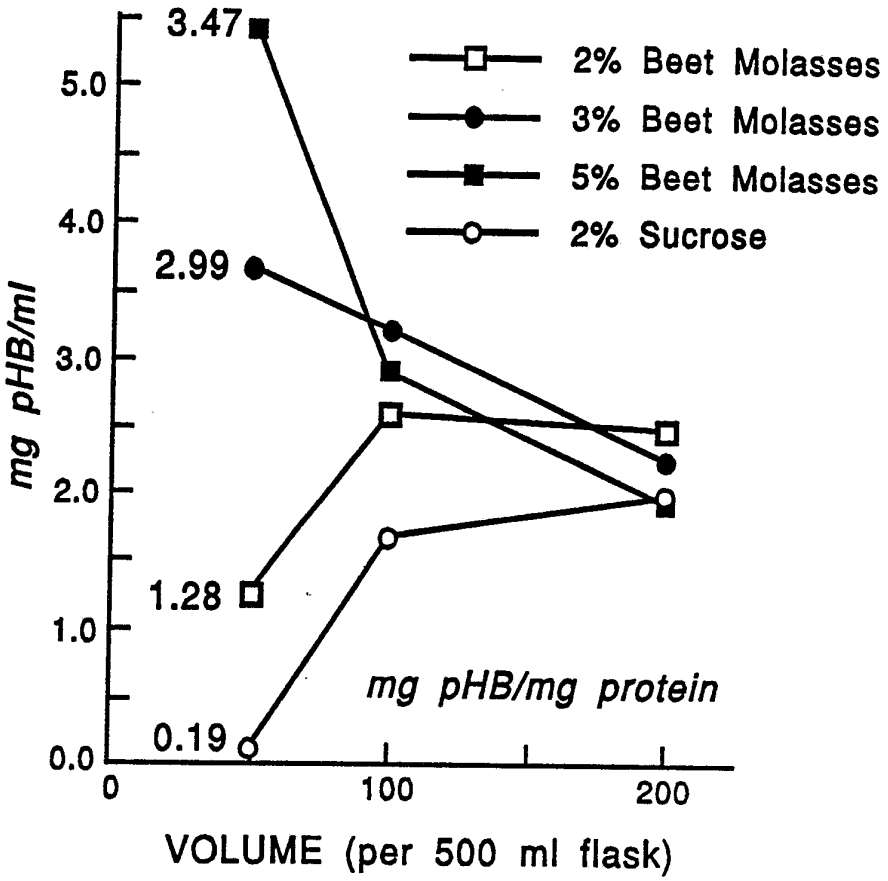

Yield pHB (mg ml-1)
2.5
2.0
1.5
1.0
0.5
0
(A)
(B)
Glucose (mg ml-1)
10
8
6
4
2
0
0
10
20
30
HOURS

FIG. 3


3.47
2.99
1.28
0.19
5.0
4.0
3.0
2.0
1.0
0.0
mg pHB/ml
0
100
200
VOLUME (per 500 ml flask)
mg pHB/mg protein
2% Beet Molasses
3% Beet Molasses
5% Beet Molasses
2% Sucrose

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 91/00183

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P7/62 ; C08G63/06

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C08G

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 005 190 (GRÜBL-KNOSP) May 17, 1990 see claims	1-10
X	CHEMICAL ABSTRACTS, vol. 104, no. 21, May 26, 1986, Columbus, Ohio, US; abstract no. 184889, MIYAMORI,TAKAO 'Production of poly(beta-hydroxybutyric acid) ' page 514 ; see abstract & JP,A,60 251 889 (MITSUBISHI) December 12, 1985	1-10
A	WO,A,8 900 202 (MASSACHUSETTS) January 12, 1989 see claims; figure 9	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 AUGUST 1991	17. 09. 91
International Searching Authority EUROPEAN PATENT OFFICE	**Signature of Authorized Officer** DELANGHE L.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9100183 SA 47781

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/09/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9005190	17-05-90	EP-A- 0441869	21-08-91
WO-A- 8900202	12-01-89	EP-A- 0329770	30-08-89