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Production of Poly-3-hydroxyalkanoates from CO and H_2 by a Novel Photosynthetic Bacterium

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ABSTRACT

A novel process is described to efficiently photoconvert low-grade organic materials such as waste biomass into natural biological plastics. When heterogeneous forms of dry biomass are thermally gasified, relatively homogeneous synthesis gas mixtures composed primarily of carbon monoxide and hydrogen are produced. Unique strains of photosynthetic bacteria were isolated that nearly quantitatively photoassimilate the carbon monoxide and hydrogen components of synthesis gas into new cell mass. Under unbalanced culture conditions when cellular growth is limited by shortages of nitrogen, calcium, magnesium, iron, or essential vitamins, up to 28% of the new cell mass is found as granules of poly-3-hydroxyalkanoate (PHA), a high-molecular-weight thermoplastic that can be solvent-extracted. The dominant monomeric unit of PHAs is 3-hydroxybutyrate (3HB), which is polymerized into the homopolymeric poly-3-hydroxybutyrate (PHB). PHB is marketed as a biodegradable plastic with physical properties similar to polystyrene. When a green alga was cocultured with the photosynthetic bacterium in light-dark (day-night) cycles, the bacteria synthesized a polymer of poly-3-hydroxybutyrate-3-hydroxyvalerate (PHB-V) with a composition of 70% 3HB and 30% 3-hydroxyvalerate (3HV) to an extent of 18% of the new cell mass. PHB-V is commercially marketed as Biopol and has physical properties similar to polypropylene or polyethylene. Our results demonstrate that a strain of photosynthetic bacteria capable of photoassimilating synthesis gas or producer gas is a potential candidate for large-scale production of biological polyesters.

Index Entries: Biodegradable plastics; poly-3-hydroxyalkanoates; PHB-V; *Rhodobacter*; carbon monoxide.

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INTRODUCTION

In recent years, microbial poly-3-hydroxyalkanoate (PHA) polymers have attracted increased industrial attention as natural thermoplastics owing to their biodegradable and biocompatible properties. In 1981, Holmes et al. (*1*) of Imperial Chemical Industries (ICI) developed a process that yielded a poly-3-hydroxybutyrate-3-hydroxyvalerate (PHB-V) copolymer from *Alcaligenes eutrophus* growing on glucose and propionic acid. The PHB-V biopolymer is marketed under the trade name of Biopol. Although poly-3-hydroxybutyrate (PHB) has physical properties similar to polystyrene, PHB-V resembles polypropylene or polyethylene (*2*). Propionic acid was essential as a precursor for the 3-hydroxyvalerate (3HV) subunits. To produce bioplastics on a commercial scale, the cost of microbial growth substrates becomes a critical factor. Use of inexpensive biomass materials would improve the economics of producing bioplastics.

Photosynthetic bacteria can entirely assimilate a wide variety of organic substrates driven by the energy of sunlight and, therefore, are a feasible host for this process if an inexpensive carbon substrate is available. Photosynthetic bacteria are known to accumulate PHAs as intracellular carbon storage materials (*3,4*). PHA synthesis normally prevails as a result of nutrient imbalance, i.e., when the exogenous supplies of carbon and energy are abundant, yet growth is limited because of the depletion of some essential nutrient (*5*).

No previously known microbes will convert lignocellulosic materials directly into new cell mass at a reasonable rate and yield. However, thermal gasification of low-grade heterogeneous organic materials produces a relatively homogeneous gaseous product primarily consisting of CO and H_2 (synthesis gas), making the bulk of the energy of the lignocellulosic materials immediately accessible in simple form. Gasification in an air-blown atmosphere generates a gas mixture composed mainly of CO, H_2, and N_2 (producer gas), which also is of potential use to capable bacteria.

Uffen (*6*) previously described a novel isolate of the photosynthetic bacterium *Rhodocyclus gelatinosus* that would grow in the dark with CO as the sole source of carbon and the energy substrate. However, the conversion efficiency of CO into cell mass was low in the dark, and the bacteria did not grow photosynthetically on CO. We report here on a subclass of photosynthetic bacteria that is able to nearly quantitatively assimilate in light the CO and H_2 components of synthesis gas or producer gas into new cell mass. Under nutrient-unbalanced growth conditions, a major portion of the cell mass occurs as PHB or PHB-V. This technique allows conversion of low-cost, thermally gasified biomass into high-value PHB and PHB-V bioplastics at a high rate and yield in a solar-driven process.

The objectives of the present work are:

1. To demonstrate the photosynthetic growth of *Rhodobacter* sp. CBS with CO as the carbon source;

2. To optimize culture conditions to enhance PHB and PHB-V yield from a CO and H_2 gas mixture; and
3. To devise the framework for practical and economical means to produce microbial PHB and copolymers from synthesis and producer gases.

MATERIALS AND METHODS

Enrichment and Isolation of Microorganisms

Soil and water samples were enriched for photosynthetic bacteria capable of utilizing CO by exposing the enrichment cultures to CO as the major source of reductant and carbon in the light. A pure bacterial strain, identified as *Rhodobacter* sp. CBS by spectral and nutritional properties (*7*), was obtained from a parking lot soil enrichment culture and used for all experiments.

Media and Growth Conditions

The basal medium was RCVBN minimal medium without either a carbon or a nitrogen source (*7*). Ten-milliliter portions of the medium were dispensed into Neoprene rubber-stoppered anaerobe tubes (25-mL volume; Bellco Glass, Inc., Vineland, NJ) under streaming N_2 or Ar using the method described by Hungate (*8*). The medium was then supplemented with $NaHCO_3$ to a final concentration of 0.1%. A reducing solution was injected by syringe to give a final concentration of 0.5 m*M* cysteine and 0.3 m*M* Na_2S; this removed traces of oxygen before sterilization in an autoclave press (Bellco Glass, Inc.). With NH_4Cl as the nitrogen source, the N_2 in the culture gas phase was replaced with Ar. For successive feedings of gaseous substrates, 6 mL of a gas mixture containing 50% CO and 50% H_2 (simulated synthesis gas) was injected sterilely into culture tubes by passing the gas through an Acrodisc filter (Gelman Sciences, Ann Arbor, MI) of 0.2 μ pore size. For nitrogen-fixing cultures, the initial gas phase concentrations of CO and H_2 were approx 15% each with the balance as N_2, which provided the only source of medium nitrogen (simulated producer gas). Producer and synthesis gases were generated from the flaming pyrolysis of red alder pellets in a laboratory-scale, downdraft gasifier operated in an air-blown or oxygen-blown mode, respectively.

Culture tubes were laid on their sides with an overhead illumination (75 W/m^2 light intensity) from incandescent lamps and shaken at 20 rpm in a rotary shaker to facilitate mass transfer of gaseous substrates into the aqueous phase. Day-night cycles of 12 h light and 12 h darkness were controlled by ChronTrol timers (CT models, Lindburg Enterprises, Inc., San Diego, CA).

Determination of Growth and Cell Dry Weight

Cell growth was monitored by measuring the culture turbidity at 660 nm with a Spectronic 21 colorimeter (Bausch & Lomb, Inc., Rochester, NY). Cell dry weight was determined by washing the centrifuged cells once with distilled water and drying them in a 100°C oven.

Analytical Procedures

To quantify the PHA and polysaccharide contents, cultures in the stationary phase of growth were harvested and analyzed according to the method described by Law and Slepecky (*9*) and by anthrone reaction (*10*), respectively.

Gases were analyzed by injecting aliquots from culture head spaces into a gas chromatograph (Varian, model 3700, Sugarlane, TX) equipped with a molecular sieve 5A column and a thermal conductivity detector. Argon was used as the carrier gas.

The nuclear magnetic resonance spectra of PHB-V samples in $CDCl_3$ were recorded at ambient temperature on a Varian Unity 300 spectrometer operated at 300 MHz with a 18.5-μs pulse width and 200-scan accumulations. TMS was the internal standard.

RESULTS

Photoassimilation of Carbon Monoxide into Cell Mass

Under anaerobic conditions, *Rhodobacter* sp. CBS performs a water-gas shift reaction with the approximate stoichiometry of: $CO + H_2O \rightarrow CO_2 + H_2$. Figure 1A shows the CO consumption with concomitant H_2 production in darkness, and the H_2 produced accumulates largely in the gas phase. A CO uptake rate of 27.6 μmol/h/mg cell dry wt was measured. Under anaerobic conditions in the light, CO was converted to H_2 and CO_2 at nearly the same rate as in the dark; however, the evolved H_2 and CO_2 are subsequently completely consumed from the gas phase. Figure 1B shows the time course of CO uptake, the transient H_2 production from CO, and the subsequent H_2 uptake by the bacteria in light.

Figure 2 shows photosynthetic growth of *Rhodobacter* sp. CBS upon successive feedings of CO and H_2 with 28 m*M* NH_4Cl as nitrogen nutrient. The photosynthetic culture had a cell doubling time of 1.1 d and reached a final cell concentration of 3.3 mg cell dry wt/mL culture. When the mass transfer of gaseous CO into the aqueous phase was enhanced through more vigorous agitation, a cell doubling time of 6.4 h was observed. *Rhodobacter* sp. CBS also can be cultured under an atmosphere of 70% N_2 gas as the sole source of nitrogen. The bacteria consume CO and H_2 simultaneously with fixation of N_2 and exhibit a cell doubling time of 2.2 d (Fig. 2).

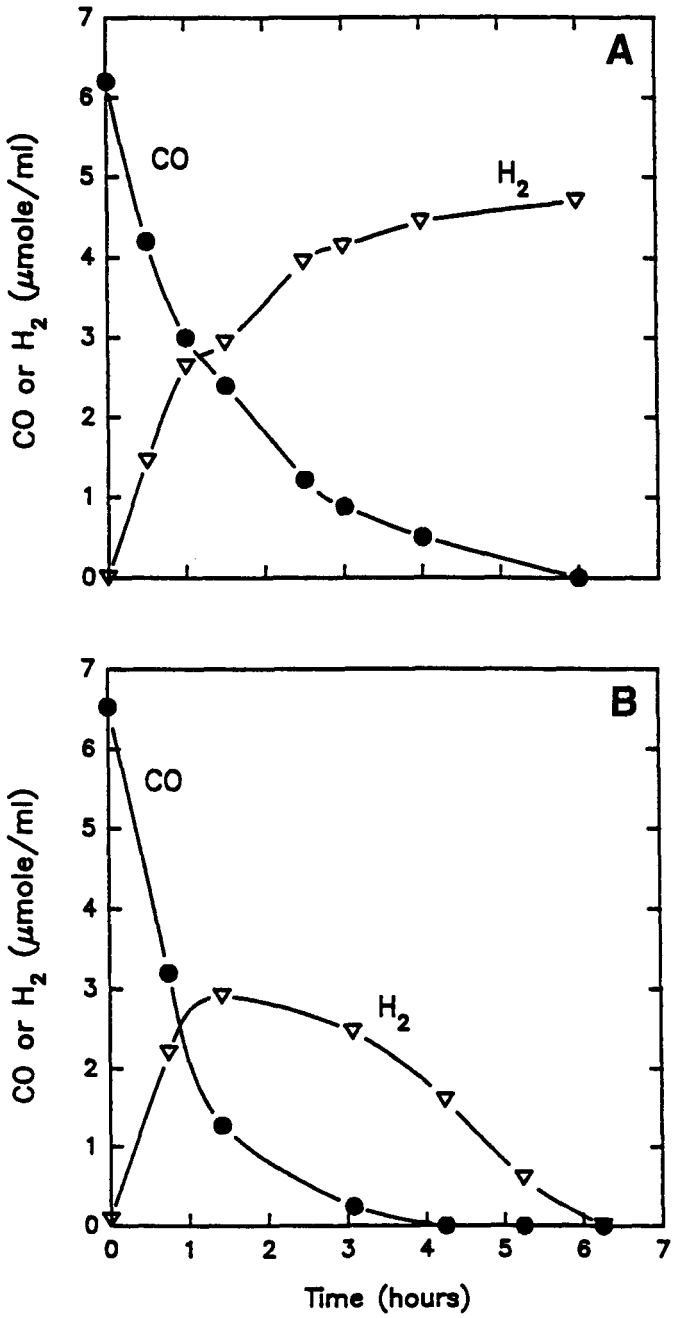

Fig. 1. Anaerobic shift of CO and H_2O to H_2 and CO_2 by *Rhodobacter* sp. CBS in (**A**) darkness and (**B**) continuous light. A culture growing photosynthetically on CO, H_2, and N_2 to logarithmic phase was used. The culture gas phase was replaced with Ar/CO_2 (80:20). Six milliliter CO was injected at time zero. At various time intervals, gas-phase samples were withdrawn and analyzed for CO and H_2 content.

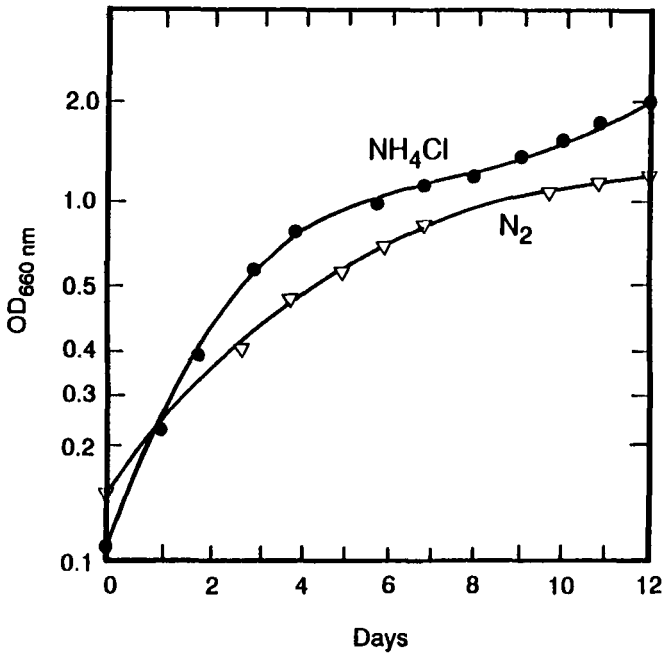

Fig. 2. Photosynthetic growth of *Rhodobacter* sp. CBS on N_2 or ammonium ion with CO as carbon substrate. NH_4Cl at 28 m*M* was supplemented to the minimal medium where indicated. Otherwise, 70% N_2 was provided as the only source of medium nitrogen.

The strain has been subcultured under these conditions for more than 1 y and remains fully active. No differences were observed when an atmosphere of water-scrubbed producer gas generated from thermally gasified woodchips was fed to the culture in place of the simulated producer gas.

A whole-cell formula of $C_5H_8O_2N$ (*11*) was used to calculate the carbon balance during growth on CO, H_2, and N_2. Approximately 94% of the available gaseous substrates were converted to cell mass with no residual gas substrates. Radiotracer experiments using ^{14}CO indicate that only 0.5% of the ^{14}C-labeled carbon was recovered as soluble extracellular organic material.

Effect of Nitrogen Limitation and Light-Dark Cycles on PHA Synthesis

Cultures of *Rhodobacter* sp. CBS were examined for their carbon storage pattern when grown anaerobically in the light with simulated producer gas. Under all growth conditions tested, the bacteria produced two major types of carbon storage materials: polysaccharide and PHA. A high content of PHB was observed when exogenous acetate was added to a stationary-phase culture growing on CO, H_2, and N_2. PHB content was 79% of the cell dry mass, setting an upper limit that potentially can be achieved

Table 1
Effect of Nitrogen Source and Light Conditions on the Production of Polymers from CO and H_2 in *Rhodobacter* sp. CBS

Sample	Nitrogen source, concentration	Light conditions	Polysaccharide, percent dry wt	PHA, percent dry wt
1a	NH_4Cl (28 m*M*)	L[a]	N.D.[b]	0.54
1b	NH_4Cl (28 m*M*)	L/D[c]	11.60	1.70
2a	NH_4Cl (1.2 m*M*)	L	33.80	8.70
2b	NH_4Cl (1.2 m*M*)	L/D	13.60	14.30
3a	N_2 (70%)	L	33.00	0.43
3b	N_2 (70%)	L/D	14.50	1.20

[a]L = continuous light at 75 w/m².
[b]N.D. = not determined.
[c]L/D = alternating 12-h light at 75 w/m² and 12-h dark period.

from the gaseous substrates alone. Photosynthetic growth in continuous light on H_2 and CO resulted primarily in polysaccharide synthesis, accounting for more than 30% of the cell dry weight (Table 1). With nonlimiting amounts of nitrogen available to the cells (28 m*M* NH_4Cl or excess N_2), less than 1% of the cell mass was found to be PHA. However, when only a limiting amount of NH_4Cl (1.2 m*M*) was present, production of PHA from CO was substantially increased to 8.7% of the cell dry weight at the expense of cellular protein.

The effect of a light-dark cycle on polymer production was also examined. When cultures were subjected to a 12-h light/12-h dark cycle, the assimilation of CO and H_2 into PHA showed overall two- to threefold increases with approx 50% decreases in polysaccharide levels compared to identical cultures incubated with continuous light. A dark period apparently promotes conversion of polysaccharide into PHA. Combining both nitrogen limitation and light/dark cycles on a single culture, 14.3% of the cell dry weight was observed to be PHA.

Effect of Other Nutrient Deficiency on Growth and PHA Synthesis

Rhodobacter sp. CBS was cultured in media in which one or more essential nutrients was eliminated. The cultures were subjected to 12-h light/12-h dark cycles with CO, H_2, and N_2 gases in a mineral medium. Table 2 lists the cell growth and polymer content under these conditions. Growth (as measured by an increase in optical density) was slowed on all occasions where one or more essential nutrients became depleted from the medium, although H_2 and CO continued to be consumed. The increases in cell mass at this stage were found to occur primarily within the PHA fraction. When a combination of nutrients was eliminated at the same time, PHA amounted to nearly 30% of the cell dry mass.

Table 2
Effect of Medium Component Deficiency on Growth and Polymer Production from CO and H_2 in *Rhodobacter* sp. CBS

Deficient component	Final OD_{660}	Polysaccharide, percent dry wt	PHA, percent dry wt
None	1.55	14.50	1.20
Basal salt[a]	0.57	27.80	7.20
Vitamin	0.83	14.30	7.10
Phosphate[b]	0.41	25.70	9.30
Sulfate	1.00	17.80	6.50
Basal salt, [a], vitamins, and trace elements	0.74	13.30	28.20

[a]The basal salt fraction contains $MgSO_4$, $CaCl_2$, and $FeSO_4$ salts.
[b]HEPES buffer, Na salt at 10 m*M* was supplemented in the medium to maintain pH at 7.0.

Cocultures of Algae and Photosynthetic Bacteria

Data in Table 1 suggest that dark incubation triggers the fermentation of endogenous polysaccharides to organic acids, which are then assimilated in a period of light into PHA. Breakdown of polysaccharide also is stimulated by a low concentration of oxygen, such as that generated by algae. A coculture of *Rhodobacter* sp. CBS and a green alga isolated from local pond water, identified as a *Chlorella* species, was developed by inoculating approx 0.5% (v/v) of the *Chlorella* species with *Rhodobacter* sp. CBS into a fresh RCVBN basal medium. When the coculture was exposed to a 12-h light/12-h dark cycle in the presence of CO, H_2, and N_2, the PHA content was elevated to 18% of cell dry weight. The assimilation of CO into cell mass by the photosynthetic bacteria was not affected by the presence of green algae in this coculture (data not shown). Green algae accounted for less than 0.5% of the culture population, probably owing to their slow growth rate and inability to fix nitrogen.

The bioplastic polymer synthesized by this coculture was extracted in chloroform and analyzed by nuclear magnetic resonance. The spectrum is shown in Fig. 3. In addition to the well-characterized resonance of methyl (carbon 4) protons in the 3HB unit (Fig. 3A), a second methyl (carbon 9) proton resonance appears at 0.89 ppm as a triplet (Fig. 3B) because of linkage to a methylene group. This linkage is characteristic of an ethyl side chain of a 3HV unit. Based on the integration ratio of the methyl resonances, a polymer of the PHB-V type with a composition of 70 mol% 3HB and 30 mol% 3HV was obtained.

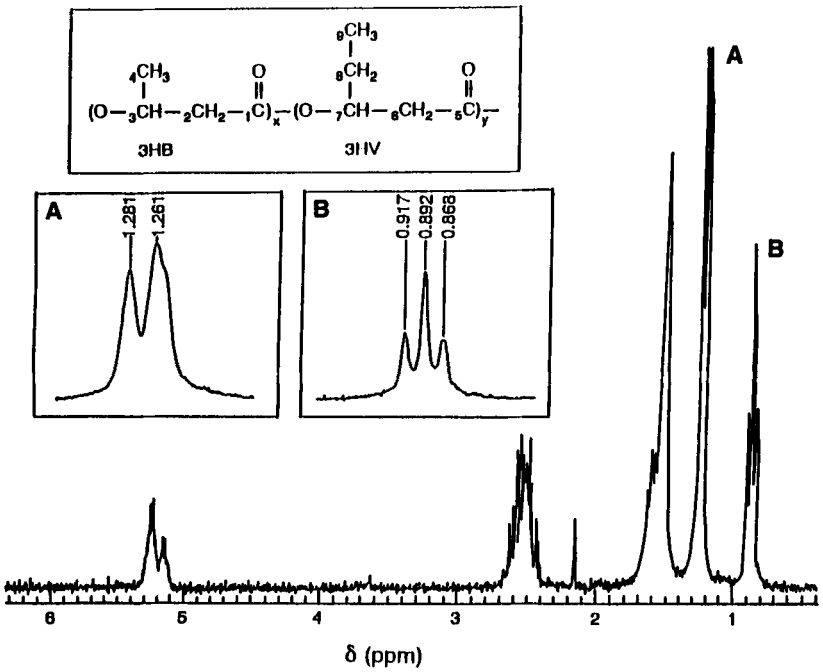

Fig. 3. 300 MHz NMR spectrum of PHB-V copolymer in $CDCl_3$.

DISCUSSION

The results of our study demonstrate that a newly isolated strain of photosynthetic bacteria is able to grow and convert the CO and H_2 components of synthesis or producer gases into a variety of PHA bioplastics that possess potential commercial applications. That this unique growth mode can be sustained for long periods of time as evidenced by the fact that a photosynthetic culture of *Rhodobacter* sp. CBS has been maintained for more than one year by repeated transfer into media containing only CO and H_2 as the major growth nutrients.

Only a few bacteria are reported to metabolize CO, including carboxydotrophic aerobes and some acetogenic, methanogenic, or sulfate-reducing anaerobes (*12*). Some isolates of photosynthetic bacteria have been reported to be capable of growing on CO as both a carbon and energy source in darkness (*13,14*). A different photosynthetic bacterium, *Rhodospirillum rubrum* S1, exhibits CO oxidation activity with a stoichiometric yield of H_2 as long as another fixed organic carbon source is available for growth (*15,16*, Maness and Weaver, unpublished data). However, the H_2 produced from CO remains accumulated in the culture gas phase without being photoassimilated into cell mass. In contrast, *Rhodobacter*

sp. CBS quantitatively consumed the CO in light after first converting it to H_2 and CO_2. This bacterium also can quantitatively consume the CO and H_2 components of a thermally generated synthesis or producer gas generated from biomass. This type of utilitarian, CO-consuming photosynthetic organism may play an important ecological role in soil or aquatic environments.

Hwang et al. (*17*) have reported that in *Azotobacter vinelandii* and *Clostridium pasteurianum*, CO is a noncompetive inhibitor and H_2 a competitive inhibitor for the nitrogenase enzyme complex. Both CO and its metabolic product H_2 normally inhibit N_2 fixation, which is an energy-intensive process. The presence of the gaseous inhibitors during growth further explains the slower growth rates observed with cultures growing on CO, H_2, and N_2. CO was reported to be an inhibitor for the uptake hydrogenase enzyme, although to a much lesser degree (*18*). Uptake hydrogenase activity is essential for the photoassimilation of H_2 and CO_2 into new cell mass. However, in *Rhodobacter* sp. CBS, the enzymes responsible for CO oxidation reactions are highly active. The initial enzyme, CO dehydrogenase, can oxidize CO (to methyl viologen) at a rate of 5 mol/h/mg enzyme. Thus, the cellular concentration of CO probably is kept well below the K_i level in order for both nitrogenase and uptake hydrogenase enzymes to be functional.

It is known that the yield of PHA and its monomeric units are related to amounts and types of organic acids present in the medium (*3,4*). Substrates that are convertible to acetate, propionate, or butyrate without the intermediate formation of pyruvate lead mostly to PHA synthesis (*5*). Indeed, our results showed that adding exogenous acetate to cultures growing on CO and H_2 increased the PHA content to 79% of cell dry mass. Currently, this is probably an impractical solution for mass production of PHA unless a source of waste acetate becomes available. Yet it does set an upper limit on the PHA level that the *Rhodobacter* bacteria can potentially produce from the gaseous substrates alone.

Formation of PHA in *Rhodobacter* sp. CBS also is stimulated by nutrient limitations. Limitations in nitrogen, basal salts (calcium, magnesium, and iron ions), vitamins, phosphate, or sulfate in the medium all significantly enhanced PHA synthesis from CO and H_2. The highest level of PHA was found in a culture where a combination of nutrients were limited. These results are similar to those obtained for PHA formation in various organisms (*19–20*).

Photosynthetic growth of *Rhodobacter* sp. CBS in continuous light on CO and H_2 substrates produces polysaccharide as the major carbon storage material. Anaerobic dark incubation induces the fermentative metabolism of photosynthetic bacteria (*21*). With the purple-sulfur photosynthetic bacteria *Chromatium vinosum*, a mobilization of polysaccharide carbon into PHA carbon occurred when the culture was exposed to alternating light and dark periods (*11,22*). The disappearance of polysaccharide in darkness coincided with the synthesis of PHA. van Gemerden et al. (*23*) also

observed the diurnal cycle of polysaccharide-to-PHB conversion in purple-sulfur photosynthetic bacteria in stratified lakes during field studies. In both cases, the researchers proposed that the oxidized and reduced components of the sulfur cycle played a major role in the observed polysaccharide breakdown to PHB synthesis in darkness and its reversal in light. Indeed, when *Rhodobacter* sp. CBS was subjected to light/dark cycles, the assimilation of CO and H_2 into PHA was significantly increased. Under this condition, the polysaccharide content showed an approx 50% decrease, presumably reflecting its conversion into PHA polymers and precursors. This finding presents a practical means to increase PHA yield, as working photobiological reactors will utilize normal solar cycles.

To broaden the potential applications of PHA synthesis from CO and H_2, polymers other than PHB need to be synthesized in a controlled manner. Different strains of photosynthetic bacteria ferment sugars into different organic acid products in darkness (*21*). For example, a *Rhodobacter capsulatus* strain produces primarily acetate and butyrate fermentation products that would be reassimilated in the light, primarily into PHB. A strain of *Rhodospirillum rubrum*, however, ferments sugars primarily into acetate and propionate products that would be reassimilated into a PHB-V polymer in light.

Breakdown of endogenous polysaccharide also is stimulated by low concentrations of oxygen. In the photosynthetic bacteria/green algae coculture experiment, we were able to maintain constant but low concentrations of oxygen in the coculture medium by limiting fixed-nitrogen levels, which limited the numbers of green algae. This facilitated the glycolytic breakdown of sugars and polysaccharides into organic acid products, including acetic and propionic acids. The PHB-V type copolymer produced in this manner amounted to 18% of cell dry mass with a composition of 70% 3HB and 30% 3HV.

Commercial costs for bioplastics could be significantly decreased by using photosynthetic bacteria, sunlight, and inexpensive synthesis gas or producer gas derived from waste organic materials. Because few bacteria can grow on CO, H_2, and N_2, sterility of the system probably is not a requirement, which should further reduce operating costs. Better understanding of the metabolic control of PHA synthesis, growth conditions that lead to higher PHA yield, and improved photosynthetic isolates should aid in the development of this incipient technology.

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