ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 3, pp. 318–323. © Pleiades Publishing, Ltd., 2008.
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EXPERIMENTAL ARTICLES

# Synthesis of Reserve Polyhydroxyalkanoates by Luminescent Bacteria

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Received February 13, 2007; in final form June, 19, 2007

**Abstract**—The ability of marine luminescent bacteria to synthesize polyesters of hydroxycarboxylic acids (polyhydroxyalkanoates, PHA) as reserve macromolecules was studied. Twenty strains from the collection of the luminescent bacteria CCIBSO (WDCM839) of the Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, assigned to different taxa (*Photobacterium leiognathi, Ph. phosphoreum, Vibrio harveyi*, and *V. fischeri*) were analyzed. The most productive strains were identified, and the conditions ensuring high polymer yields in batch culture (40–70% of the cell dry mass weight) were determined. The capacity for synthesizing two- and three-component polymers containing hydroxybutyric acid as the main monomer and hydroxyvaleric and hydroxyhexanoic acids was revealed in *Ph. leiognathi* and *V. harveyi* strains. The results allow luminescent microorganisms to be regarded as new producers of multicomponent polyhydroxyalkanoates.

*Key words*: luminescent bacteria, polyhydroxyalkanoates (PHA), biosynthesis, chemical structure.

**DOI:** 10.1134/S0026261708030119

Analysis of the literature suggests active development of the works aimed at synthesizing and studying polymers based on carboxylic acid derivatives. Along with polylactides and polyglycolides, polyhydroxyalkanoates (PHA), microbiologically produced polymers of hydroxy fatty acid derivatives, occupy a special place among biodegradable polyesters. The most important feature of PHA is that biosynthesis can produce polymers of various chemical structures with different properties, from highly crystalline thermoplastics to rubbery elastomers with a low melting temperature [1, 2]. The ability to accumulate PHA of different structure depends on the physiological and biochemical characteristics of producer strains and the conditions of their growth. Different approaches may be used to increase the production and synthesis of new types of PHA, including the isolation of new environmental producer strains, modification of the nutrient media composition and the cultivation conditions, as well as constructing genetically modified strains [3, 4].

Despite the increasing number of recent publications devoted to PHA and a large number of articles on the biochemistry and genetics of the system of PHA synthesis, as well as the description of new producers and the processes of PHA accumulation, our knowledge of the regulation of the mechanisms of synthesis of these macromolecules is highly limited [5–7]. In the opinion of the leading investigators of this process [6–9], the regulation of PHA synthesis may be carried out at different levels: (1) by activating the expression of *pha* genes by such specific environmental factors as deficiency of biogenic elements (e.g., nitrogen or phosphorus); (2) by regulating the activity of the key enzymes of PHA synthesis at the substrate level; and (3) by a combination of both factors. The report that the luminescent *Vibrio harveyi* synthesizes polyhydroxy butyrate (PHB), the most widespread PHA, and PHB synthesis in dense culture of these bacteria is controlled and regulated by N-(3-hydroxybutanoyl)-homoserine lactone, an autoinducer of the luminescence process, is highly important for extending the concept of the biochemical regulation of the process of PHA synthesis [10].

This report is very valuable for several reasons. First, this is the first experimental proof of the regulation of the process of PHA synthesis by the cell metabolism regulator N-(3-hydroxybutanoyl)-homoserine lactone, which is an autoinducer of luminescence; second, this is proof of the simultaneous involvement of acyl homoserine lactones in the regulation of more than one metabolic process; and third, this is evidence of the possibility of PHA synthesis by luminescent bacteria. It should be noted that in none of the available fundamental reviews dedicated to the diversity of PHA producers (of which over 300 are presently known) were luminescent bacteria regarded as potential producers of these macromolecules.

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The data available on the possibility of PHA synthesis by luminescent bacteria of the genus *Vibrio* and the existence of common pathways for the regulation of light generation and of PHA accumulation [10, 11] determined the goals of this work, in which luminescent bacteria were studied as a new potential PHA producer and for possible sources of new types of polymers.

## MATERIALS AND METHODS

The strains from the collection of luminescent bacteria CCIBSO (WDCM839) of the Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, were studied [12]. The capacity for PHA synthesis was assessed in 20 strains (Table 1), including nine *Photobacterium leiognathi* strains, eight *Vibrio harveyi* strains, two *Ph. phosphoreum* strains, and one *V. fischeri* strain.

For primary screening, Egorova's agarized medium was used [13] containing the following (g/l): NaCl, 30; $MgSO_4 \cdot 7H_2O$, 0.5; $KH_2PO_4$, 1; asparagine, 2.5; peptone, 10; agar-agar, 18; fish extract, 0.5 l; distilled water, 0.5 l. The petri dishes were inoculated with the museum strains; the cultivation was carried out at 25°C for 24 h.

To grow the bacterial strains in batch suspension culture, we used medium of the following composition (g/l): NaCl, 30; $MgSO_4 \cdot 7H_2O$, 0.2; $KH_2PO_4$, 1; $Na_2HPO_4 \cdot 12H_2O$, 6; and peptone (the main growth substrate), 0.5 (in special experiments the peptone concentration was varied). This medium differed from the conventional one [14] in the absence of $(NH_4)_2HPO_4$ (0.5 g/l), which was only added in the course of initial optimization of the conditions for increasing PHA production. Glycerol (3 g/l) or glucose (1 g/l) were used as additional carbon sources. The nitrogen content in peptone, as determined using a Flash EA 1112 CNH element analyzer (Neolab, Italy), was 75 g/kg with a moisture content of 4.7%. Batch cultivation was carried out in 500-ml flasks with a culture volume of 250 ml at 28°C on a thermostatically controlled shaker. The medium was inoculated with the biomass washed off from agarized museum cultures with 3% NaCl.

In the course of cultivation of luminescent bacteria, the optical density of suspensions was monitored with a KFK-2 photoelectric colorimeter at $\lambda = 540$ nm in 3-mm cuvettes. The biomass yield (g/l) was determined using the calibration graph of a relationship between the culture optical density and the cell concentration. The intensity and dynamics of luminescence were measured using a BLM-8802 bioluminometer (SKTB Nauka, Krasnoyarsk Scientific Center, Siberian Branch, Russian Academy of Sciences) at a sensitivity of $10^6$ quanta/s. The device was calibrated by the Hastings & Weber standard [15]. In order to assess the level of the luminescent reaction, the specific luminescence intensity (the ratio of the luminescence intensity of a bacterial culture (quanta/$s^{-1}$) to its biomass) was calculated.

**Table 1.** The studied strains of luminescent bacteria

| Species | Strain | Source of isolation | Region of isolation |
|---|---|---|---|
| *Ph. leiognathi* | 208 | Seawater | Pacific Ocean |
| | 307 | Seawater | Pacific Ocean |
| | 543 | Stomach of the fish *Sumbolophorus rufinus* | Indian Ocean |
| | 683 | Stomach of the fish *Diaphus lucidus* | Indian Ocean |
| | 1504 | Seawater | Indian Ocean |
| | 1612 | Seawater | Indian Ocean |
| | 1680 | Seawater | Indian Ocean |
| | 1759 | Gut of the cuttlefish *Sepia confusa* | Indian Ocean |
| | 2117 | Seawater | Indian Ocean |
| *Ph. phosphreum* | 1856 | Light organ of the fish *Opisthoproctus soleatus* | Indian Ocean |
| | 1883 | Light organ of the fish *Opisthoproctus soleatus* | Indian Ocean |
| *V. fischeri* | 1231 | ND | ND |
| *V. harveyi* | 72 | Seawater | Pacific Ocean |
| | 162 | Seawater | Pacific Ocean |
| | 328 | Seawater | South China Sea |
| | 767 | Surface of the mollusk *Conus ebraeus* | Arabian Sea |
| | 974 | Holothurian (*Holothurioidea*) stomach | Indian Ocean |
| | 1024 | Mollusk (*Gastropoda*) gut | Indian Ocean |
| | 1175 | Gut of the mollusk *Nerita albicilla* | South China Sea |
| | 2303 | Seawater | Sea of Japan |

Note: ND means that no data are available.

The bacterial biomass grown on solid media was washed off with 3% NaCl solution. The biomass washed off from solid media, as well as the biomass obtained in batch cultures, was centrifuged at 6000 rpm, with the medium residues washed off twice with distilled water and dried for 24 h at 105°C. After methanolysis of the biomass samples, the PHA intracellular concentration and chemical structure were determined by chromatography of fatty acid methyl esters [16] on the GCD plus chromato mass spectrometer (Hewlett Packard, United States). The lipids and

**Table 2.** PHA production on solid media by luminescent bacteria (HB, β-hydroxybutyrate; HV, β-hydroxyvalerate)

| Species | Strain | PHA content, % of the dry matter | PHA composition, mol % |
|---|---|---|---|
| *Ph. leiognathi* | 208 | 18.1 | HB, 100% |
| | 307 | 5.0 | HB, 100% |
| | 683 | 10.4 | HB, 99.13%; HV, 0.87% |
| | 1504 | 0.4 | HB, 100% |
| | 1612 | 0.4 | HB, 100% |
| | 1680 | 1.7 | HB, 100% |
| | 1759 | 0.7 | HB, 100% |
| | 2117 | 1.2 | ND |
| *Ph. phosphoreum* | 1856 | 4.0 | HB, 100% |
| | 1883 | 7.4 | HB, 100% |
| *V. fischeri* | 1231 | 0.5 | ND |
| *V. harveyi* | 72 | 4.8 | HB, 99.25%; HV, 0.75% |
| | 162 | 0.5 | ND |
| | 328 | 0.5 | HB, 100% |
| | 767 | 0.6 | ND |
| | 974 | 0.7 | ND |
| | 1024 | 0.8 | ND |
| | 1175 | 1.1 | ND |
| | 2303 | 0.9 | HB, 100% |

Note: ND stands for "no data."

the polymer were extracted from the biomass with a mixture of chloroform and ethanol (2 : 1 vol/vol). The polymer was separated from the lipids by precipitation with a double volume of hexane and rinsed with hexane. For the methanolysis of the polymer samples, 1 ml of the internal standard (0.5 mg benzoic acid/1 ml chloroform), 0.85 ml of methanol, and 0.15 ml of concentrated sulfuric acid were added to the weighed polymer sample (4 g) and boiled with backflow condensers for 2h 40 min. Upon completion of the methanolysis, a double volume of distilled water was added to the flask.

## RESULTS

The capacity for PHA synthesis on solid media was noted for all the strains studied (Table 2); however, the polymer yields differed significantly and constituted from 0.4 to 18.1% (of the dry matter weight). The maximum content of the polymer was recorded in *Ph. leiognathi* 208. Mass spectral analysis showed that the polymer synthesized by most strains on a solid medium was homogeneous and consisted of the β-hydroxybutyric acid monomer. The presence of β-hydroxyvaleric acid in PHA, along with hydroxybutyrate, was detected in two strains only (*Ph. leiognathi* 683 and *V. harveyi* 72).

The strain *Ph. leiognathi* 208 was used for the subsequent experiments as the most PHA-productive.

The dynamics of polymer accumulation by the strain was studied on medium with peptone and glycerol. The main characteristics of the *Ph. leiognathi* 208 culture grown in batch mode on a standard medium with $(NH_4)_2HPO_4$ are presented in figure a. Since the PHA yields were insignificant, the medium composition was further adjusted, and ammonium hydroorthophosphate was excluded from it. The results of cultivation of the strain on the medium containing 0.5 g/l of peptone (respective nitrogen content was 38 mg/l) and 3 g/l of glycerol are presented in figure b. During the first 8 h after inoculation, the luminescence intensity increased together with the biomass increase with the luminescence peaking (about $10^{10}$ billion (quanta/s)/(g/l biomass)) after 8 to 10 h of cultivation (figure b). Not until the luminescence of the growing culture decreased dramatically did the polymer accumulate in the cells; its concentration at the early stationary phase reached 37.7%, while the biomass yield was 100 mg/l.

The highest level of luminescence in luminescent cultures is known to be observed at high culture density, i.e., under nonoptimal growth conditions [17, 18]. An increase in the intracellular PHA pool also occurs under nonoptimal conditions; in the case of batch culture conditions, it means the end of the linear and the beginning of the stationary growth phase. Under these conditions accompanied by the emerging deficiency of biogenic elements, as well as the accumulation of cell metabolites, the rate of the synthesis of the major nitrogen-containing cell macromolecules decreases with a resultant increase in the concentration of reducing elements, which, in turn, inhibit citrate synthase and isocitrate dehydrogenase of the tricarboxylic acid cycle [19]. This leads to decreased respiration (which was also shown for *Ph. leiognathi* [17]) and a smaller free coenzyme A content in the cells, which also favors PHA synthesis. A discrepancy in time between the processes of luminescence and polymer accumulation in the cultures of luminescent bacteria may results from the competition for reduced pyridine nucleotides (NADH and NADPH) between the luminescence system and the system of PHA synthesis, which may cause an increase in the polymer content with a decrease in luminescence. The competition of the two systems for the fatty acids used for synthesizing both PHA monomers (hydroxycarboxylic acids) and long-chain aldehydes, the luciferase reaction substrates, is also possible. Therefore, active PHA accumulation observed in the culture of luminescent bacteria after the luminescence decline seems to be connected with the intracellular pool of the fatty acids being redirected under these conditions from the bioluminescence pathway to the system of polymer synthesis.

It is known from the literature that, for most of the PHA producers characterized to date, except *Alcaligenes latus*, considerable accumulation of the polymer occurs under unbalanced growth. The conditions stim-

ulating PHA synthesis are species-specific; for some producers, it is the deficiency of biogenic elements (nitrogen, phosphates) in the medium, while for others it is oxygen limitation [19]. Such information is not available in to the case of luminescent bacteria. Therefore, to determine the conditions stimulating the accumulation of PHA in *Ph. leiognathi*, we varied the medium parameters, including peptone concentration (from 0.5 to 15 g/l), nitrogen supply to the culture, the presence of an additional source of carbon (glycerol or glucose), and medium salinity in the course of bacterial growth.

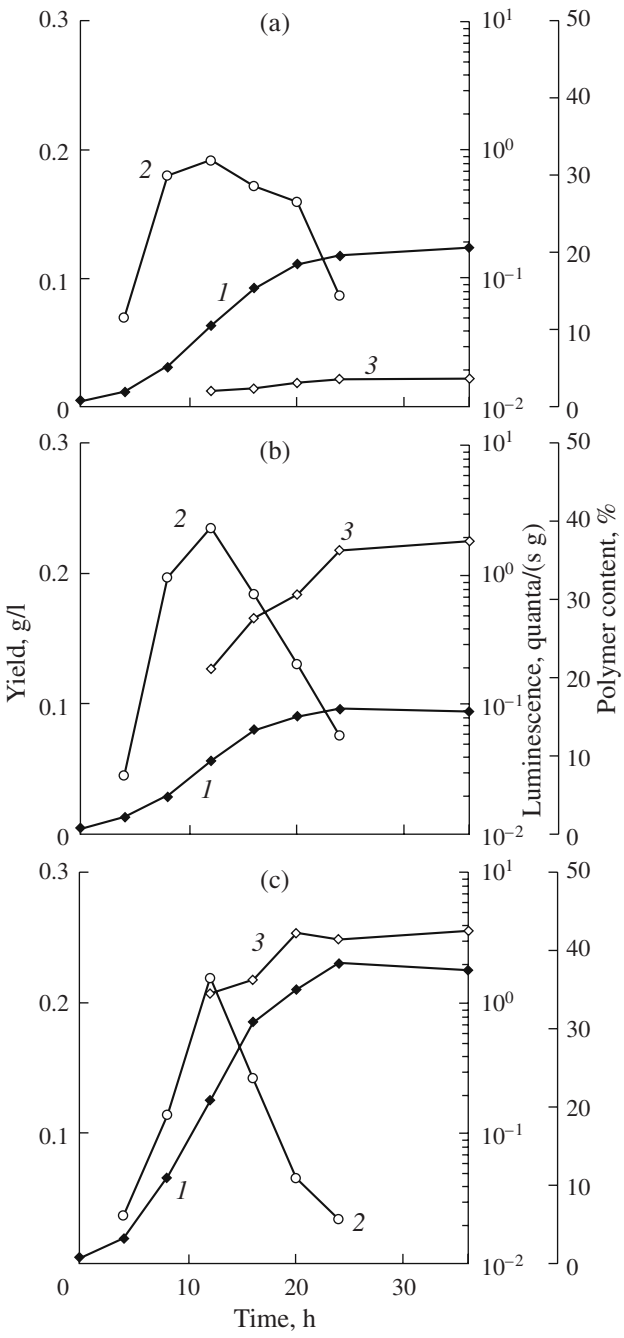
An increase in peptone concentration from 0.5 to 1.0 g/l in a medium with glycerol (figure b, c) significantly increased the biomass yield in the culture and had a positive effect on the polymer output, whose content in the biomass attained 42.5% (Table 3). With peptone concentration in the medium increased to 5 g/l (respective nitrogen content was 375 mg/l), the bacterial yield increased to 920 mg/l; however, the polymer concentration decreased to 22.2%. When nitrogen was introduced into the medium in the form of $(NH_4)_2HPO_4$ (0.5 g/l), the polymer synthesis decreased dramatically (to 3.7%). Thus, to achieve high PHA synthesis, the growth of luminescent bacteria should be nitrogen-limited. A peptone concentration of 15 g/l inhibited bacterial growth and PHA synthesis; no influence on luminescence was revealed.

The substitution of glucose for glycerol, irrespective of the peptone concentration in the medium, did not stimulate the bacterial growth and had a negative effect on the polymer synthesis (Table 3). The cell yield in the culture and the polymer output did not exceed 300 mg/l and 17.5%, respectively. These results were obtained on the medium containing 1 g/l of glucose and 1 g/l of peptone.

A decrease in the medium salinity (sodium chloride concentration was decreased from 3%, as is customary for this species, to 1%) had a negative effect on the yield, the polymer synthesis, and the culture luminescence.

Thus, the highest PHA yields (37–42%) in the culture of *Ph. leiognathi* 208 were obtained on the medium with peptone (0.5–1.0 g/l) and glycerol (3 g/l), although at a comparatively low cell yield. This is a consequence of the specificity of the process of synthesis of PHA, which are reserve macromolecules within the cells; this process occurs actively only when microbial growth is unbalanced. For this reason, simultaneously obtaining high PHA yields and high biomass production is problematic [4]. It should be noted that these results were obtained on the physiologically inactive strains stored in the museum. Subsequent cultivation of these microorganisms with multiple transfers will probably allow us to increase the physiological activity and to obtain more productive forms.

The ability of individual organisms to synthesize heteropolymeric PHA consisting of short-, medium-, or




Dynamics of growth, bioluminescence, and polymer accumulation in *Ph. leiognathi* 208: (a) peptone, 0.5 g/l; $(NH_4)_2HPO_4$, 0.5 g/l; (b) peptone, 0.5 g/l; and (c) peptone, 1 g/l. Yield (g of dry biomass/l) (*1*); luminescence (quanta/(s g of dry biomass)) (*2*); PHA content (% of dry biomass) (*3*).

long-chain-length monomers with the $C_3$–$C_4$–$C_5$ (hydroxy derivatives of propionate, butyrate, and valerate), $C_6$–$C_{12}$ (hydroxy derivatives of hexanoate, octanoate, nonanoate, etc.), and $C_6$–$C_{12}$ chain length, respectively, is known from the literature [20]. Few publications on the polymer production by luminescent bacteria available report only detection of homoge-

**Table 3.** The polymer content and composition in the cells of *Ph. leiognathi* 208 as depended on the cultivation conditions (HB, β-hydroxybutyrate; HV, β-hydroxyvalerate; HH, β-hydroxyhexanoate)

| Peptone concentration, g/l | Additional carbon source | PHA content, % of the dry matter | Biomass yield, g/l | PHA output, g/l | PHA composition, mol % |
|---|---|---|---|---|---|
| 0.5 | Glucose | 9.6% | 0.13 | 0.01 | HB, 100%; HB, traces |
| 1 | Glucose | 17.5% | 0.30 | 0.05 | HB, 99.9%; HB, 0.1% |
| 5 | Glucose | 7.8% | 0.92 | 0.07 | HB, 98.3%; HB, 1.2%; HH, 0.5% |
| 15 | Glucose | 0.69% | 0.31 | 0.002 | HB, 100% |
| 0.5 | Glycerol | 37.7% | 0.10 | 0.04 | HB, 99.3%; HB, 0.4%; HH, 0.3% |
| 1 | Glycerol | 42.5% | 0.23 | 0.10 | HB, 99.5%; HB, 0.2%; HH, 0.3% |
| 5 | Glycerol | 22.2% | 0.92 | 0.20 | HB, 100%; HB, traces; HH, traces |
| 15 | Glycerol | 2.75% | 0.58 | 0.02 | HB, 100% |

neous polyhydroxybutyrate at a concentration not exceeding 13% [10, 21, 22].

The polymer synthesized by *Ph. leiognathi* 208 on the medium containing peptone and glycerol (or glucose) as the carbon substrate without supplemented hydrocarbon acids (valerate, hexanoate, etc.) inducing the synthesis of heteropolymeric PHA [16] contained hydroxyvalerate and hydroxyhexanoate monomers as minor components (Table 3). This allows this strain to be considered not only as a potential polyhydroxybutyrate producer but also a multi-component PHA producer.

Seven more strains of luminescent bacteria were introduced into the culture, namely, representatives of *Ph. leiognathi*, *V. harveyi, V. fischeri*, and *Ph. phosphoreum* (Table 4). They were chosen for further studies as the most active polymer producers (Table 2) after analyzing their PHA content on solid media. On media with peptone and glycerol (5 and 3 g/l, respectively), all the strains showed a considerable increase in the intracelluar PHA concentration, except *V. fischeri* and *Ph. phosphoreum*, whose accumulation of PHA in the periodic culture was at a level of an experiment on solid media. The highest yields of the polymer were recorded for *Ph. leiognathi*, with strains 543 and 683 showing very significant yields (47.1 and 71.0%). The analysis of mass spectra showed the PHA synthesized by *Ph. leiognathi* 208, 543, and 683, as well as by *V. harveyi* 72, to be multicomponent and to contain hydroxybutyrate as the dominant monomer with minor inclusions of hydroxyvalerate and hydroxyhexanoate.

Thus, it is the first time that luminescent bacteria have been analyzed as potential PHA producers. The most productive strains were isolated, and the conditions providing for a sufficiently high polymer output in batch culture were determined. The capacity of synthesizing two- and three-component polymers, which is significant for the biotechnology of PHA, was revealed in the representatives of *Ph. leiognathi* and *V. harveyi*.

**Table 4.** Production of PHA by luminescent bacteria in batch culture (5 g/l of peptone and 3 g/l of glycerol in the medium) (HB, β-hydroxybutyrate; HV, β-hydroxyvalerate; HH, β-hydroxyhexanoate)

| Species | Strain | PHA content, % of the dry matter | PHA yield, g/l | PHA composition, mol % |
|---|---|---|---|---|
| *Ph. leiognathi* | 208 | 22.2 | 0.20 | HB, 100%; HV, traces; HH, traces |
| | 307 | 0.2 | <0.001 | HB, 100% |
| | 543 | 47.1 | 0.62 | HB, 99.8%; HB, 0.2% |
| | 683 | 71.0 | 1.36 | HB, 99.3%; HB, 0.5%; HH, 0.2% |
| *Ph. phosphoreum* | 1856 | 5.1 | 0.014 | HB, 100% |
| | 1883 | 1.3 | <0.002 | HB, 00% |
| *V. fischeri* | 1231 | 0.4 | 0.003 | HB, 100% |
| *V. harveyi* | 72 | 12.3 | 0.20 | HB, 99.2%; HB, 0.4%; HH, 0.4% |

## ACKNOWLEDGMENTS

The work was supported by the Russian Federation Ministry of Education and Science and the U.S. Civilian Research and Development Foundation, project no. RUX0-002-KR-06; the Russian Science Support Foundation (the program "The RAS Candidates and Doctors of Science"); the Krasnoyarsk Regional Scientific Foundation, project no. 16G146; Russian Foundation for Basic Research (project no. 07-03-0112) and the integration project no 24, Siberian Branch, Russian Academy of Sciences.

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