Luminous Bacteria as Producers of Polyhydroxyalkanoates

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Summary: The study addresses the ability of luminous bacteria of different taxa (*Photobacterium leiognathi, Photobacterium phosphoreum, Vibrio harveyi, Vibrio fischeri)* to synthesize polyesters of hydrocarbon acids (polyhydroxyalkanoates, PHAs) as storage macromolecules. The screened strains widely varied in their PHA productivity. Conditions for attaining high polymer yields (including two- and three-component polymers) in batch culture have been determined. The attained polymer yields reached 40-70% of dry cell biomass. The results suggest a conclusion that luminous microorganisms can be considered as producers of multi-component PHAs.

Keywords: luminous bacteria; polyhydroxyalkanoates; polyhydroxybutyrate

Introduction

Polyhydroxyalkanoates (PHAs) – microbial polymers of hydroxy fatty acids – have received much attention recently for potential applications in various spheres. The greatest advantage of PHAs is that biosynthesis can yield polymers of various chemical structures, exhibiting different properties – from high-crystallinity thermoplastic polymers to rubber-like elastomers.[1–2] To increase PHA production and to create new types of PHAs, scientists isolate new PHA producers, modify culture conditions, and construct genetically modified strains.[1,3]

Although there are more than 300 known PHA producers described in a number of fundamental reviews, no mention of luminous bacteria as potential producers of these macromolecules has been made. However, there are several publications on polymer production by luminous bacteria which report determination of intracellular homogeneous polyhydroxybutyrate.[4–6] Moreover, it has been reported that the *luxR* gene product of *Vibrio harveyi* does not only regulate bioluminescence but also stimulates PHA synthesis. [4,7] The maximal luminescence level is registered after luminous bacteria reach nonoptimal growth conditions (high biomass density).[8] PHA accumulation also occurs when conditions are nonoptimal. The presence of the common gene-regulator of these processes can be indicative of their common regulation. Using reducing equivalents (PHA synthesis and bioluminescence involve NADH and $FADH_2$) and fatty acids (used for synthesis of PHA monomer units and bioluminescence substrates – long-chain aldehydes) can also result in interaction between two biochemical systems. Thus, the purpose of this study was to investigate luminous bacteria as a novel potential PHA producer.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

Strains of the species *Photobacterium leiognathi*, *Ph. phosphoreum*, *Vibrio harveyi* and *V. fischeri* from Collection CCIBSO 836 (WDCM 836) of the Institute of Biophysics (Siberian Branch of Russian Academy of Sciences), Krasnoyarsk, Russia, were

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Table 1.
Screened strains of luminous bacteria and their PHA production on solid media (HB – β-hydroxybutyrate, HV – β-hydroxyvalerate).

Species	Strain	Isolation area	PHA content, %	PHA composition
Photobacterium leiognathi	208	The Pacific Ocean	18.1	HB – 100%
	307	The Pacific Ocean	5.0	HB – 100%
	543	The Indian Ocean	2.8	HB – 100%
	683	The Indian Ocean	10.4	HB–99.1%; HV–0.9%
	1504	The Indian Ocean	0.4	HB – 100%
	1612	The Indian Ocean	0.4	HB – 100%
	1680	The Indian Ocean	1.7	HB – 100%
	1759	The Indian Ocean	0.7	HB – 100%
	2117	The Indian Ocean	1.2	N.D.
Photobacterium phosphoreum	1856	The Indian Ocean	4.0	HB – 100%
	1883	The Indian Ocean	7.4	HB – 100%
Vibrio fischeri	1231	N.D.	0.5	N.D.
Vibrio harveyi	72	The Pacific Ocean	4.8	HB–99.3%; HV–0.7%
	162	The Pacific Ocean	0.5	N.D.
	328	The South China Sea	0.5	HB – 100%
	767	The Arabian Sea	0.6	N.D.
	974	The Indian Ocean	0.7	N.D.
	1024	The Indian Ocean	0.8	N.D.
	1175	The South China Sea	1.1	N.D.
	2303	The Japanese Sea	0.9	HB – 100%

investigated.[9] Twenty strains were screened for their ability to synthesize PHAs: 9 strains of *Photobacterium leiognathi*, 8 *Vibrio harveyi*, 2 *Photobacterium phosphoreum*, and 1 *Vibrio fischeri* (Table 1).

For the primary screening, these strains were plated in Petri dishes on Egorova's Fish-peptone agar medium (water fish extract 500 mL, NaCl 30.0 g, peptone 10.0 g, KH_2PO_4 1.0 g, $MgSO_4$ 0.5 g, agar-agar 18.0 g, H_2O up to 1 L). Water fish extract was prepared by boiling pike-perch (600 g) in water (1000 mL) and filtration. The culture was conducted at 25 °C for 24 h. Bacteria were grown in batch suspension culture, using a semi-synthetic medium (g/L) (NaCl 30.0 g, KH_2PO_4 1.0 g, $Na_2HPO_4 12H_2O$ 10.0 g, $MgSO_4$ 0.2 g, glycerol 3.0 g, and peptone in different (0.5 to 15 g/L) concentrations). $(NH_4)_2HPO_4$ (0.5 g/L) was sometimes added during primary optimization experiments. Nitrogen contained in peptone was measured using a Flash EA 1112 CHN elemental analyzer ("Neolab", Italy) and amounted to 75 g/kg at 4.7% moisture. Bacteria were batch cultured in 500 mL flasks containing 250 mL culture at temperature 28 °C on an incubator shaker. Biomass washed off agar cultures with a 3% NaCl solution was inoculated into the medium.

Biomass Yield and Bioluminescence Measurements

Optical density of bacterial suspension was measured using a KFK-2 photoelectric colorimeter at 540 nm, with optical path length 3 mm. Biomass yield (g/L) was determined using the standard curve of culture optical density versus cell concentration. The intensity and the dynamics of culture luminescence were measured using a BLM-8802 bioluminometer (Special Engineering and Design Department "Nauka" at the Krasnoyarsk Research Center SB RAS) of sensitivity 10^6 quanta·sec^{-1}. The bioluminometer was calibrated using the standard of Hastings and Weber.[10] To determine the level of luminescent reaction, specific luminescence (luminescence intensity of bacterial culture (quanta $\cdot$ sec^{-1})/biomass of the culture) was calculated.

Transmission Electron Microscopy

Cells were washed, fixed in 2.5% glutaraldehyde, encapsulated in agar (1%), post-fixed in a 1% osmium tetroxide solution for

3 h, dehydrated in graded ethanol solutions, and embedded in epoxy resins, an Epon-Araldite mixture (Serva, Germany). Ultrathin sections were cut with a glass knife on a Reichert OMU3 ultramicrotome, additionally contrasted with a 0.2% aqueous solution of lead isocitrate, and examined under a JEM-100C electron microscope (JEOL, Japan) at an accelerating voltage of 80 KV.[11]

Analysis of Accumulated PHAs

Bacterial biomass grown on agar media was washed off with a 3% NaCl solution. The washed-off biomass and the batch-grown biomass were centrifuged at 6000 rpm and washed twice with a 3% NaCl solution; then, biomass was dried at 105 °C for 24 h. The PHA concentration and composition were determined by chromatography of fatty acid methyl esters on a GCD-Plus gas chromatograph-mass spectrometer (GC-MS) (Hewlett Packard) after acid-catalyzed (H_2SO_4) methanolysis of a dry biomass sample (4 mg). Benzoic acid was used as the internal standard.[12]

Results and Discussion

All studied strains were found to be able to synthesize PHAs on solid media (Figure 1), but polymer content in the cells varied. Polymer yields ranged from 0.4 to 18.1% of dry matter weight (Table 1). The largest polymer content was in *Ph. leiognathi* 208 cells. Analysis of mass spectra showed that the polymer synthesized by most of the strains on agar medium was homogeneous and consisted of a monomer of β-hydroxybutyric acid. Only two strains (*Ph. leiognathi* 683 and *V. harveyi* 72) also contained some amounts of β-hydroxyvaleric acid.

In the subsequent experiments *Ph. leiognathi* 208 was used as the most efficient of the tested PHA producers. Dynamics of polymer accumulation by this strain was investigated. The main characteristics of *Ph. leiognathi* 208 batch cultured in semi-synthetic medium containing 0.5 g/L of peptone (38 mg/L of nitrogen) and 0.5 g/L $(NH_4)_2HPO_4$ are shown in Figure 2a. As just insignificant PHA yields were obtained, a modified medium, which did not contain ammonium hydro-orthophosphate was used. Results of growing the strain on this modified medium are shown in Figure 2b. During the first 8 h following inoculation, culture luminosity increased along with the biomass, with the luminosity reaching its maximum after 8–10 h of culture. Then, luminosity of the culture dropped drammatically and only after that the polymer accumulated in the cells, reaching 37.7% by the onset of the stationary phase, with biomass yield amounting to 100 mg/L.

The maximum luminescence level is registered after luminous bacteria reach

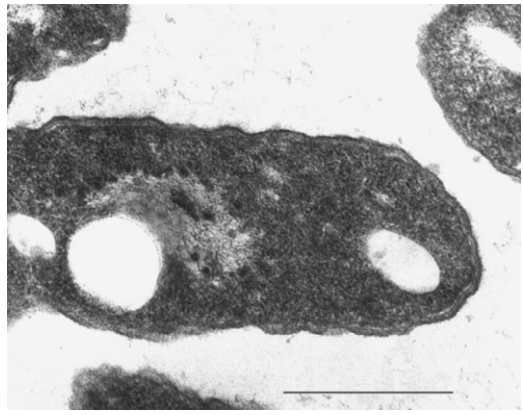

Figure 1.
Ultrathin section of a *Ph. leiognathi* cell with polyhydroxybutyrate granules. Scale line length is 0.5 μm.

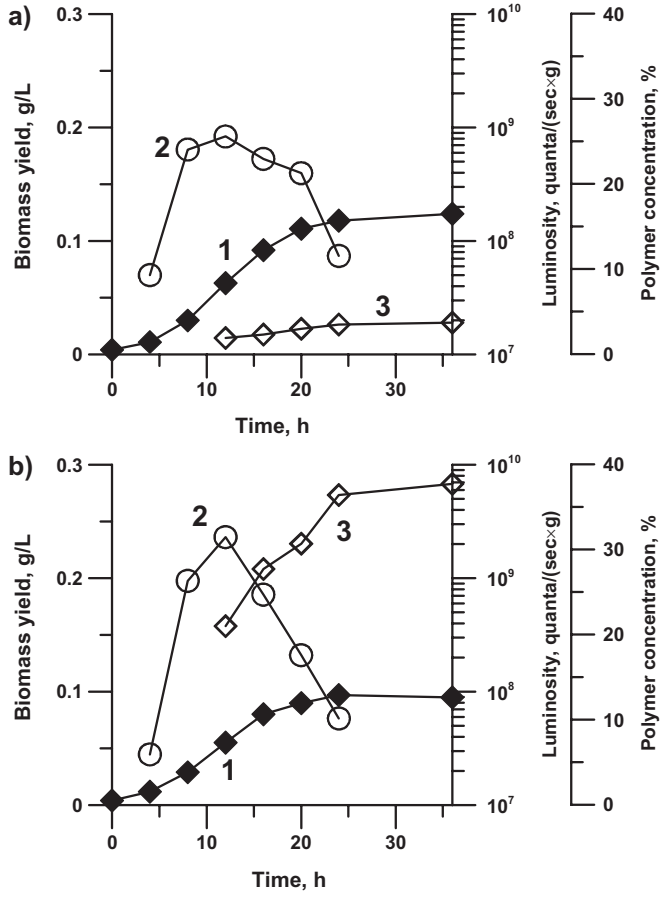

Figure 2.
Dynamics of *Ph. leiognathi* 208 growth, bioluminescence and polymer accumulation in medium containing 0.5 g/L peptone: a) medium with $(NH_4)_2HPO_4$; b) medium without $(NH_4)_2HPO_4$. Notations: 1 – yield, 2 – luminosity, 3 – PHA concentration in biomass.

high biomass density, i.e. under nonoptimal growth conditions.[13] Intracellular PHA pool also grows under nonoptimal conditions, which in batch culture generally develop at the end of the linear growth phase and beginning of the stationary one. Under these conditions, when biogenic elements become deficient and metabolic products of the cells build up, synthesis of the major nitrogen-containing cellular macromolecules slows down, leading to higher concentrations of reducing equivalents and, thus, inhibition of citrate synthase and isocitrate dehydrogenase of the tricarboxylic acid cycle.[14] As a result, respiration decreases (which was also shown for *Ph. leiognathi*[15]) and the cellular level of free coenzyme A is depleted, which is also favorable for PHA synthesis. The fact that bacterial luminescence and intracellular polymer accumulation in the culture of luminous bacteria occur at different times may be accounted for by competition for pyridine nucleotides (NAD · H and NADP · H) between luminescent system and PHA synthesis system, which can be responsible for polymer accumulation under decreased luminescence. The two systems may also compete for fatty acids used in synthesis of both hydroxycarboxylic

acids – PHA monomers – and long-chain-length aldehydes – substrates of luciferase reaction. Thus, rapid PHA accumulation registered in the culture of luminous bacteria after luminosity decline may have resulted from the intracellular pool of fatty acids being redirected from the bioluminescent pathway to the polymer synthesis system.

Analysis of the literature shows that most of the PHA producers that have been studied by now, except *Alcaligenes latus*, accumulate significant amounts of the polymer under unbalanced growth. Factors inducing PHA synthesis are species specific; for some PHA producers this is deficiency of biogenic elements (nitrogen, phosphates) in the medium whereas for others this is oxygen deficiency.[14] Such factors for luminous bacteria have so far remained unknown. Thus, to find the conditions inducing PHA accumulation in *Ph. leiognathi* cells, during culture we varied such parameters of the medium as peptone concentration (0.5 to 15 g/L), nitrogen supply, and salinity of the medium.

A peptone concentration increase from 0.5 g/L to 1.0 g/L considerably enhanced the biomass yield and favorably affected accumulation of the polymer, whose concentration in the biomass reached 42.5%. When peptone concentration was increased to 5 g/L (375 mg/L of nitrogen), bacterial biomass yield reached 920 mg/L, but polymer concentration dropped to 22.2%. Nitrogen added to the medium as $(NH_4)_2HPO_4$ (0.5 g/L) dramatically reduced polymer synthesis (to 3.7%). It can be concluded that nitrogen deficiency induces luminous bacteria to synthesize PHAs. The peptone concentration 15 g/L inhibited both bacterial growth and PHA synthesis; luminescence was not affected.

A decrease in sodium chloride concentration of the medium from 3% (generally accepted concentration for this species) to 1% adversely affected biomass growth, polymer synthesis, and luminosity of the culture.

Thus, the largest PHA yields (37–42%) in *Ph. leiognathi* 208 culture were obtained using the medium containing 0.5–0.1 g/L peptone; the end cell concentration was, however, relatively low. This is due to the specific nature of the synthesis of PHAs, which are cell storage macromolecules and are synthesized in considerable amounts only under unbalanced growth of microorganisms. Hence, it would be problematic to obtain both high PHA and high biomass yields.[3] We should note that these results were obtained in experiments with physiologically inactive strains after long storing on solid nutrient media. Further cultures of these organisms, involving repeated inoculations, may enhance their physiological activity and yield more productive forms.

As reported in the literature, there are microorganisms that can synthesize heteropolymer PHAs consisting of various-chain-length monomers, containing 3 to 12 carbon atoms.[16] The few available studies on polymer production by luminous bacteria report determining intracellular homogeneous polyhydroxybutyrate (PHB) only, at concentrations not higher than 13%.[4–6]

The polymer synthesized by *Photobacterium leiognathi* 208 on the medium that contained peptone and glycerol as carbon substrates but was not supplemented with hydrocarbon acids, inducing synthesis of heteropolymer PHAs (valerate, hexanoate, etc.) [17], contained minor amounts of hydroxyvalerate and hydroxyhexanoate monomers (Table 2). So, this strain can be considered as a potential producer of both polyhydroxybutyrate and multi-component PHAs.

We cultured 7 more strains of luminous bacteria – representatives of *Ph. leiognathi, V. harveyi, V. fischeri* and *Ph. phosphoreum*, which were selected for investigation after the primary screening on agar media as the most efficient PHA producers (Table 2). In the medium containing 5 g/L peptone all strains exhibited a significant increase in intracellular PHA concentration, except *V. fischeri* and *Ph. phosphoreum*, which accumulated as much PHA as they did on agar medium. The highest polymer yields were registered for *Ph. leiognathi*, for strains 543 and 683 in particular – 47.1% and 71.0%.

Table 2.
PHA production by batch cultured luminous bacteria.[a)]

Species	Strain	PHA content, % dry matter	PHA yield, g/L	PHA composition, mol%
Photobacterium leiognathi	208	22.2	0.20	HB – 100%; HV – traces; HHx - traces
	307	0.2	<0.001	HB – 100%
	543	47.1	0.62	HB – 99.8%; HV – 0.2%
	683	71.0	1.36	HB – 99.3%; HV – 0.5%; HHx – 0.2%
Photobacterium phosphoreum	1856	5.1	0.014	HB – 100%
	1883	1.3	<0.002	HB – 100%
Vibrio fischeri	1231	0.4	0.003	HB – 100%
Vibrio harveyi	72	12.3	0.20	HB – 99.2%; HV – 0.4%; HHx – 0.4%

HB – β-hydroxybutyrate, HV – β-hydroxyvalerate, HHx – β-hydroxyhexanoate.
[a)]in the medium containing 5 g/L of peptone and 3 g/L of glycerol.

Analysis of mass spectra showed that PHAs synthesized by *Ph. leiognathi* 208, 543 and 683 and by *V. harveyi* 72 were multicomponent, containing hydroxybutyrate as a major monomer and hydroxyvalerate and hydroxyhexanoate as minor ones.

Conclusion

In this work luminous bacteria were for the first time analyzed as potential PHA producers. The most productive strains were selected and conditions for obtaining fairly high polymer yields in batch culture were determined. It was found that representatives of *Ph. leiognathi* and *V. harveyi* are able to synthesize two- and three-component polymers – a significant finding for PHA biotechnology.

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