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Production of Two- and Three-Component Polyhydroxyalkanoates by Luminous Bacteria of the *Photobacterium* Genus

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The study addresses the ability of luminous bacteria Photobacterium leiognathi Boisvert et al. and Photobacterium phosphoreum (Cohn) Beijerinck to synthesize polyesters of hydroxycarbon acids (polyhydroxyalkanoates, PHAs) as storage macromolecules. The screened strains widely vary in their PHA productivity. Ph. leiognathi (but not Ph. phosphoreum) produces PHAs containing monomers of 3-hydroxyvaleric acid and, in some cases, 3-hydroxyhexanoic acid, in addition to common monomers of 3-hydroxybutyric acid. All studied strains of Ph. phosphoreum produce pure poly-3-hydroxybutyrate only. In the case of Ph. leiognathi, addition of valeric acid as substrate can increase the amounts of medium-chain-length hydroxy acids contained in the produced polymers. The results suggest a conclusion that luminous microorganisms of Photobacterium genus can be considered as producers of multi-component PHAs.

Keywords: luminous microorganisms, Photobacterium, polyhydroxyalkanoates, polyhydroxybutyrate.

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Продукция двух- и трехкомпонентных полигидроксиалканоатов светящимися бактериями рода *Photobacterium*

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Исследована способность светящихся бактерий видов Photobacterium leiognathi Boisvert et al. и Photobacterium phosphoreum (Cohn) Beijerinck к синтезу полиэфиров гидроксикарбоновых кислот (полигидроксиалканоатов – ПГА) в качестве резервных макромолекул. Исследованные штаммы значительно различались по их способности к синтезу ПГА. Ph. leiognathi (но не Ph. phosphoreum) синтезировал ПГА, которые, помимо мономеров поли-3-гидроксибутирата, содержали мономеры 3-гидроксивалериановой и, иногда 3-гидроксикапроновой кислот. Все изученные штаммы Ph. phosphoreum образуют только гомополимерный поли-3-гидроксибутират. В случае Ph. leiognathi добавление валериановой кислоты в качестве субстрата может увеличивать содержание 3-гидроксивалериановой и 3-гидроксикапроновой кислот в полимере. Результаты позволяют рассматривать светящиеся микроорганизмы рода Photobacterium в качестве продуцентов многокомпонентных ПГА.

Ключевые слова: светящиеся микроорганизмы, Photobacterium, полигидроксиалканоаты, поли-3-гидроксибутират.

Introduction

Polyhydroxyalkanoates (PHAs) – microbial reserve polymers of hydroxy fatty acids – are synthesized by microorganisms under unbalanced growth (e.g., in the presence of carbon source and under deficiency of nitrogen or oxygen). PHAs serve as energy store and help microorganisms survive under unfavorable conditions. These biopolymers 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 (Steinbüchel, 2003; Sudesh et al., 2000). To increase PHA production and to create new types of PHAs, scientists isolate new PHA producers, modify culture conditions, and construct genetically modified strains (Madison, Huisman, 1999; Volova, 2004).

Polyhydroxybutyrate is the most common of PHAs but the chemical compositions of PHAs vary widely. Physiological features of bacterial strains and cultivation conditions affect the composition of monomer units, which is caused by the substrate specificity of PHA-synthases (key enzymes of polymer biosynthesis) of different microorganisms. As PHAs can vary in their composition, it would be expedient to search

for PHA producers among different bacterial strains.

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, as it has been reported by some researchers, luminous bacteria can synthesize some amounts of polyhydroxybutyrate, and biochemical processes of light generation and PHA accumulation involve the use of common metabolites (Miyamoto et al., 1998; Sun et al., 1994). Thus, the purpose of this study was to investigate luminous bacteria as a novel potential PHA producer.

Materials and methods

The study microorganisms were strains of the species *Photobacterium leiognathi* Boisvert et al. and *Photobacterium phosphoreum* (Cohn) Beijerinck from Collection CCIBSO (WDCM836) (Rodicheva et al., 1997). Fifteen strains were screened for their ability to synthesize PHAs: 6 strains of *Ph. leiognathi*, and 9 strains of *Ph. phosphoreum* (Table 1).

Bacteria were stored 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 g) and filtering. In experiment bacteria were grown in batch suspension culture, using the medium of the following composition (g/L): NaCl – 30, $MgSO_4 \times 7H_2O$ – 0.2, KH_2PO_4 – 1, $Na_2HPO_4 \times 12H_2O$ – 6, peptone – 5, glycerol – 3. The medium differed from the conventional one (Farghaly, 1950) by the absence of $(NH_4)_2HPO_4$ (0.5 g/L). Valeric acid previously neutralized with potassium hydroxide was added when necessary at 1 g/L. 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. Before experiments two passages were made in batch culture. Bacteria were batch cultured in 500-ml flasks containing

Table 1. Screened strains of luminous bacteria

Species	Strain	Isolation source	Isolation area
Photobacterium leiognathi	208	Seawater	The Pacific Ocean
	231	Seawater	The Pacific Ocean
	543	Fish *Sumbolophorus rufinus*, stomach	The Indian Ocean
	544	N.D.	The Indian Ocean
	554	Seawater	The Indian Ocean
	683	Fish *Diaphus lucidus*, stomach	The Indian Ocean
Photobacterium phosphoreum	1	Type strain (ATCC 11040; NCMB 1282)	
	1694	Seawater	The Indian Ocean
	1699	Seawater	The Indian Ocean
	1812	Fish *Chlorophthalmus* sp., bowel	The Indian Ocean
	1856	Fish *Opisthoproctus soleatus*, the light organ	The Indian Ocean
	1883	Fish *Coryphaenoides serrulatus*, bowel	The Indian Ocean
	1909	Fish *Coelorhynchus fasciatus*, the light organ	The Indian Ocean
	1912	Fish *Coelorhynchus fasciatus*, bowel	The Indian Ocean
	1920	Fish *Coelorhynchus fasciatus*	The Indian Ocean

250 ml culture at temperature 28°C on an incubator shaker. The biomass washed off agar cultures with a 3% NaCl solution was inoculated into the medium.

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 batch-grown biomass was 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 hydrolysis of a dry biomass sample (4 mg) and re-esterification of fatty acids. Benzoic acid was used as the internal standard (Brandl et al., 1988).

Results and discussion

It was earlier shown that polymer accumulation by luminous bacteria occurs during the stationary growth phase, after the decline in luminosity (which, as the culture grows, passes through a latency period, a rise, which occurs when the culture reaches high density levels, and subsequent decay), and remains almost unchanged for a long time (Boyandin et al., 2007). Thus, in studies aimed at finding efficient PHA producers among luminous bacteria, one can take samples of biomass in the stationary phase, after the luminosity decay, when polymer concentration is the highest.

We studied 15 strains of luminous bacteria grown in batch culture (Table 2). PHA yields varied widely, depending on the strain. *Ph. leiognathi* strains synthesized considerable amounts of the polymer (47% and more). In the case of *Ph. leiognathi* 208, PHA yield was

Table 2. PHA production by batch-cultured luminous bacteria

Species	Strain	PHA content, % dry matter	PHA yield, g/L	PHA composition, mol. %		
				3-hydroxy-butyrate	3-hydroxy-valerate	3-hydroxy-hexanoate
Photobacterium leiognathi	208	59	1.0	99.9	0.1	n.i.**
	231	57	0.6	100	traces	n.i.
	543*	47.1	0.62	99.8	0.2	traces
	544	49	0.5	99.8	0.2	n.i.
	554	63	0.3	99.9	0.1	n.i.
	683*	71.0	1.36	99.3	0.5	0.2
Photobacterium phosphoreum	1	1	< 0.01	100	n.i.	n.i.
	1694	12	0.08	100	n.i.	n.i.
	1699	5	0.01	100	n.i.	n.i.
	1812	4	0.01	100	n.i.	n.i.
	1856*	5.1	0.014	100	n.i.	n.i.
	1883*	1.3	< 0.002	100	n.i.	n.i.
	1909	< 0.1	< 0.001	100	n.i.	n.i.
	1912	n.i.	0	n.i.	n.i.	n.i.
	1920	1	< 0.002	100	n.i.	n.i.

* Strains were studied earlier (Boyandin et al., 2007).
** not identified.

higher than obtained earlier (Boyandin et al., 2007) possibly because of additional passages of microbial cultures before experiments. *Ph. phosphoreum* strains produced much less polymer, and *Ph. phosphoreum* 1912 did not synthesize any polymer at all in our experiment.

Monomer composition of the synthesized polymers was analyzed. The polymers synthesized by five *Ph. leiognathi* strains contained quantifiable (0.1% to 0.5%) amounts on 3-hydroxyvalerate and the polymer synthesized by Strain 683 contained 0.2% of 3-hydroxycaproate (3-hydroxyhexanoate). Polymers synthesized by *Ph. phosphoreum* strains contained one component only – 3-hydroxybutyric acid.

PHA biosynthesis in cells of microorganisms occurs through the formation of acyl coenzymes A and can at this stage incorporate short-chain-length carboxylic acids through acyl CoA and then – 3-hydroxyacyl CoA. Of major significance is substrate specificity of PHA synthase, which either allows or does not allow monomers to be incorporated into the polymer chain (Volova, 2004). In the studies involving other microorganisms it was shown that addition of carboxylic acids with carbon chains containing five and more carbons as substrate can induce incorporation of the respective 3-hydroxy acids into the PHA (Volova et al., 2007). In our experiments luminous bacteria were grown on media supplemented with valeric acid. The supplements were added to the microbial culture at different growth stages. The lower the density of the culture, the more the addition of carboxylic acids suppressed the growth of microorganisms (Fig. 1).

Having analyzed the synthesized polymers, we found that the addition of valerate in the initial stage of bacterial culture induced incorporation of the largest amounts of 3-hydroxyvalerate (Fig. 2).

To further screen the effect of valerate on the composition of the polymers produced by luminous bacteria, the substrate was added to the medium in the initial stage of the culture (during inoculation) (Table 3). In the medium supplemented with valerate, most of the study strains accumulated smaller amounts of the polymer and, as a result, their PHA yields were lower. In the *Ph. phosphoreum* 1920 culture supplemented with valerate no polymer was

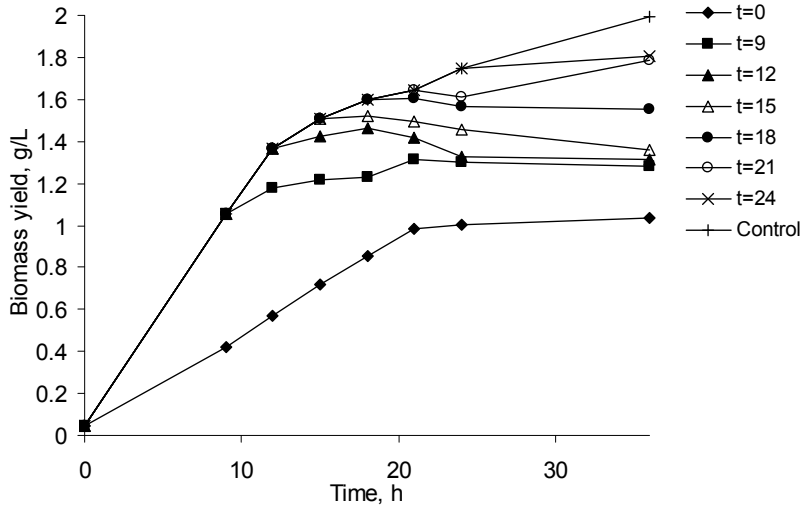

Fig. 1. Effect of valeric acid supplements on the growth of *Ph. leiognathi* 208. The legend shows the time since inoculation

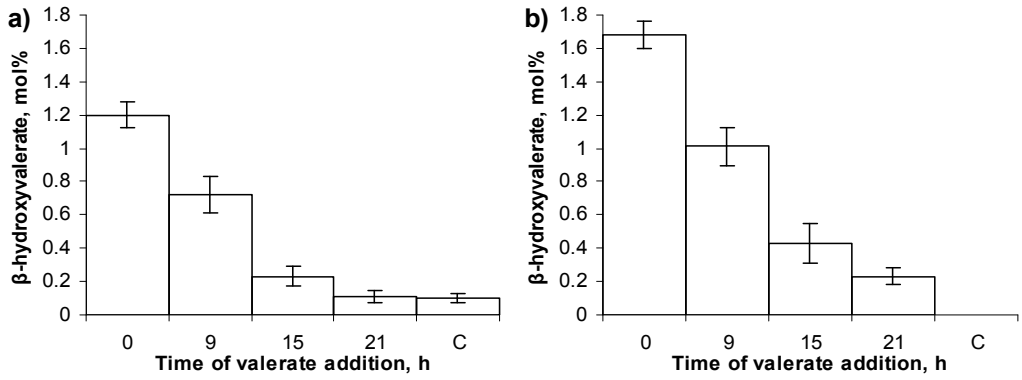

Fig. 2. Effect of valeric acid supplements on the amounts of 3-hydroxyvalerate contained in the PHAs produced by *Ph. leiognathi* strains: a) 208, b) 231, depending on the time interval between inoculation and supplementation. C – control sample (without valerate)

Table 3. PHA production by batch-cultured luminous bacteria in the medium supplemented with valerate

Species	Strain	PHA content, % dry matter	PHA yield, g/L	PHA composition, mol%		
				3-hydroxy-butyrate	3-hydroxy-valerate	3-hydroxy-hexanoate
Photobacterium leiognathi	208	51	0.4	98.75	1.2	0.05
	231	8	0.01	98.4	1.7	n.i.*
	543	4	0.01	99.3	0.7	n.i.
	544	13	0.03	99.5	0.5	n.i.
	554	0.6	< 0.002	99.9	0.1	n.i.
	683	32	0.1	99.3	0.5	0.2
Photobacterium phosphoreum	1	0.5	0.001	100	n.i.	n.i.
	1694	0.2	0.001	100	n.i.	n.i.
	1699	traces	< 0.001	100	n.i.	n.i.
	1812	0.6	0.001	100	n.i.	n.i.
	1856	0.4	0.002	100	n.i.	n.i.
	1883	0.6	< 0.001	100	n.i.	n.i.
	1909	traces	< 0.001	100	n.i.	n.i.
	1912	n.i.	0	n.i.	n.i.	n.i.
	1920	n.i.	0	n.i.	n.i.	n.i.

* not identified.

detected. *Ph. leiognathi* strains were the most stable PHA producers.

PHAs synthesized by all the study strains of *Ph. leiognathi*, except 554 and 683, in the culture supplemented with valerate contained significantly larger amounts of 3-hydroxyvaleric acid. No minor hydroxy acids were found in the PHAs produced by *Ph. phosphoreum* in experiments both with and without the addition of valerate. The addition of valerate was found to induce incorporation of 3-hydroxyhexanoic acid in the polymer produced by *Ph. leiognathi* 208.

Thus, *Ph. leiognathi* cells grown in the medium supplemented with valerate, which is

hydroxyvalerate precursor substrate, can produce polymers containing increased amounts of this hydroxy acid. In a contrast, *Ph. phosphoreum* strains were found unable to synthesize multi-component PHAs.

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