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EXPERIMENTAL ARTICLES

The Factor Stabilizing the Bioluminescence of PVA-Immobilized Photobacteria

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Abstract—Immobilization of photobacteria in the cryogel of polyvinyl alcohol (PVA) was carried out. Immobilization was found to result in increased intensity and stability of bioluminescence. The elements determining the stability of bioluminescence were investigated. Selection of the strain was found to be of the highest importance. Among immobilized cells, *Photobacterium phosphoreum* exhibited the most intense and prolonged light emission, while *Vibrio harveyi* showed the least one. The technological procedures for cryogenic immobilization of photobacteria were determined. The role of the environment of gel formation in the preservation of the bioluminescence activity was determined. In the gels formed in rich medium for submerged cultivation of photobacteria, almost 100% luminescence activity was preserved, while light emission was considerably prolonged. Bioluminescence intensity of the preparations was shown to depend significantly on pH of the incubation medium. The pH shift to acidic values during prolonged incubation of immobilized cells was shown to be one of the factors of bioluminescence quenching. The stress effects of cryogenic immobilization were found to have an insignificant effect on the temperature profile of bioluminescence. Decreased reduction rate of the luciferase flavin substrate was shown to be a possible reason for bioluminescence quenching.

Keywords: immobilization, polyvinyl alcohol, photobacteria, bioluminescence
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For application of immobilized photobacteria as toxin biosensors, strict requirements on bioluminescence duration and stability should be met (Kuts et al., 2010; Ismailov and Aleskerova, 2015). These parameters depend primarily on the emission properties of a given strain, as well as on the immobilization technology, storage procedure, and conditions of application (Lee et al., 2005; Park et al., 2005; Efremenko et al., 2014). In various strains, specific bioluminescence activity varies from 10^2 to 10^5 quanta/(s cell). In submerged culture of *Vibrio harveyi*, the luminescence cycle is short and is fully completed during the exponential growth phase (12–14 h), while in *V. fischeri* the luminescence cycle is as long as 20–24 h, and stable luminescence of *Photobacterium phosphoreum* is maintained for one to two weeks (Nealson, 1978; Kuts and Ismailov, 2009). Immobilization of bacteria was shown to result in significant prolongation of bioluminescence (Park et al., 2005; Lee, 2006; Efremenko et al., 2010). For instance, bioluminescence duration of the cells immobilized in Ca- and Sr-alginate gels increased to 2–4 weeks (Chun et al., 1996), although bioluminescence quenching was reported to be more rapid in immobilized cells than in free-living ones (Makiguchi et al., 1980).

The differences in bioluminescence stability reported in different works may be associated with the immobilization procedure and the technology for storage of the preparations. The agar, agarose, and alginate gels, which are most commonly used for immobilization of photobacteria, are formed at temperatures from 30 to 50°C, which may impair emission activity of the cells in the course of immobilization. Apart from the temperature regime, partial substitution of the Sr or Ca ions by Na ions may also affect stability of the matrix of Ca- and Sr-alginate gels under conditions of their storage and application (Chun et al., 1996). Apart from the gel-forming materials listed above, cryogels of polyvinyl alcohol (PVA) are often used for microbial immobilization (Lozinskii, 1998; Lozinsky and Plieva, 1998).

PVA cryogels are formed at negative temperatures, which is highly important in the case of the luminescence reaction readily inhibited by temperature. Formed PVA cryogels are stable within a broad range of positive temperatures (up to 80°C), which makes it possible to use the test systems in different temperature modes. Physicochemical parameters of the matrix depend but insignificantly on the chemical composition of the environment of gel formation,

including the salt composition of the medium, which is important for the bioluminescence activity of marine photobacteria. The presence of macropores (0.1–10 μm) relieves the diffusional limitations of the matrix for the substrates and for a wide range of toxins with diverse chemical structure; the latter is highly important for the application of toxicological biosensors.

The literature data on the application of this carrier for immobilization of luminescent bacteria are, however, scarce. Makiguchi et al. (1980) carried out comparative analysis of the carriers for immobilization of the luminescent bacteria *P. phosphoreum* using polyvinyl alcohol, polyurethane, polyacrylamide, Ca-alginate, and Ca-carboxymethylcellulose. The PVA-immobilized preparations of photobacteria exhibited less stable light emission compared to other carriers.

The technology for PVA immobilization of the cells of *V. fischeri* and genetically engineered *Pseudomonas putida* strain carrying the *lux* operon from *Photorhabdus luminescence* was used to obtain biofilms for detection of phenol toxins in industrial waste (Philp et al., 2003). However, glycerol was added to the imobilization mixture in order to enhance the stability of light emission. The procedure also included the stage of luminescence activation by incubation in special media.

We have developed and optimized the technological operations for immobilization of psychrophilic bacteria *P. phosphoreum* in PVA gel with almost 100% preservation of light emission, which did not require additional cryoprotectors and bioluminescence activation (Efremenko et al., 2010; Efremenko et al., 2014).

The goal of the present work was to investigate the major factors responsible for bioluminescence stabilization of the cells immobilized in PVA cryogel.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The following strains were used in the work: *Vibrio harveyi* 292 MAV ATCC, *V. fischeri* 6 KKM MGU, and two *Photobacterium phosphoreum*: strains, a moderately psychrophilic (ATCC 11040) and a psychrophilic 331 KKM MGU isolated from the White Sea.

Bacteria were grown in submerged culture on the medium containing the follwing (g/L): NaCl, 30.0; Na_2HPO_4, 5.3; $KH_2PO_4 \cdot 2H_2O$, 2.1; $(NH_4)HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract, 1.0; peptone, 5.0; glycerol, 3.0; distilled water, to 1 L; pH 7.5. The cultivation temperature was 20°C for *P. phosphoreum* strains or 25°C for *V. harveyi* and *V. fischeri*.

Biomass isolation. The cells were centrifuged at 3000 *g* for 20 min, washed with sodium phosphate buffer (pH 7.6) with 2% NaCl (pH 7.6), and centrifuged again under the same conditions. The pellet of wet biomass was used for analysis and immobilization. Late exponential-phase cultures with cell density of $2–5 \times 10^9$ cells/mL were used to obtain immobilized preparations (8 h for *V. harveyi* or 22 h for *P. phosphoreum* and *V. fischeri*).

Immobilization procedure. The cells were suspended in a solution of PVA (molecular mass 48000) to obtain a homogeneous mixture. Cell concentration in the mixture was 1%. PVA solution was prepared in the cultivation medium (CM), CM without peptone, and 3% NaCl. The mixture was distributed (200 μL) in the wells of 96-well plates, frozen at –20°C, incubated at this temperature for 17 h to obtain cryogels with the cells embedded into the polymer matrix, and stored at –80°C. The composition of the preparation was as follows (wt %): bacterial biomass, 0.62; polyvinyl alcohol, 6.8; water phase, up to 100. Thawing of the plates was carried out at 4°C for 17 h, and the granules were then stored at 4°C in 0.1 M Na-phosphate buffer with 2% NaCl.

Electron microscopy. The samples for scanning electron microscopy (SEM) were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h, dehydrated in a series of ethanol solutions of increasing concentration, in 100% acetone, dried at the critical point on Dryer HCP-2 (Hitachi, Japan), coated with gold and palladium of an IB-3 Ion Coater (Eiko, Japan), and examined under a JSM-6380LA microscope (JEOL, Japan).

Biomass determination. Cell concentrations in growing cultures or suspensions were determined spectrophotometrically as OD_{630}. The number of immobilized cells in the granules was assessed as ATP content determined by the bioluminescence method using firefly luciferase (Lundin, 2000).

Light emission activity. Bacterial bioluminescence was recorded on a 1250 LKB-Wallac luminometer and was expressed either in relative units for the routine experiments, or in absolute values of quantum yield (Q, quanta/s) using the standard of Hastings and Weber (1963).

For analysis of the bioluminescence activity in cultures or suspensions, they were diluted $10^3–10^5$ times with 3% NaCl, and the bioluminescence maximum for 10 s was measured in 0.1 M Na phosphate buffer with 2% NaCl (pH 7.6) at room temperature. Bioluminescence of immobilized preparations was determined as an emission maximum of a single granule in 1 mL of the same buffer after equilibration of the temperatures of the granules and the solution (22°C) for 1–2 min.

RESULTS

Structure of PVA preparations with photobacteria. Stability and emission activity of bacteria in a carrier are known to depend on the immobilization and reactivation procedures, storage conditions, the presence of substrates and protectors, and rheological charac-

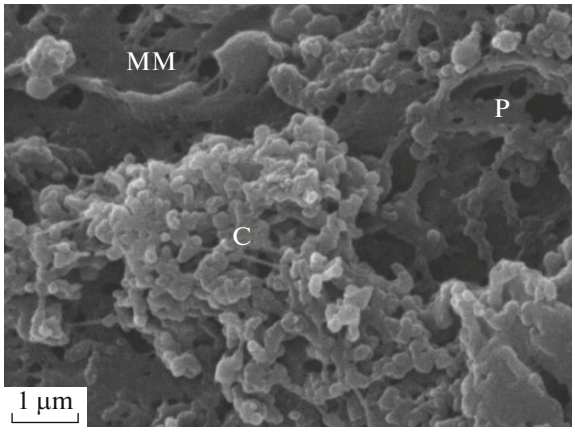

Fig. 1. *P. phosphoreum* immobilized in the matrix of polyvinyl alcohol (PVA) at 1%. Designations: C, cells; MM, mucous material; P, pores. Scanning electron microscopy (SEM). Scale bar, 1 μm.

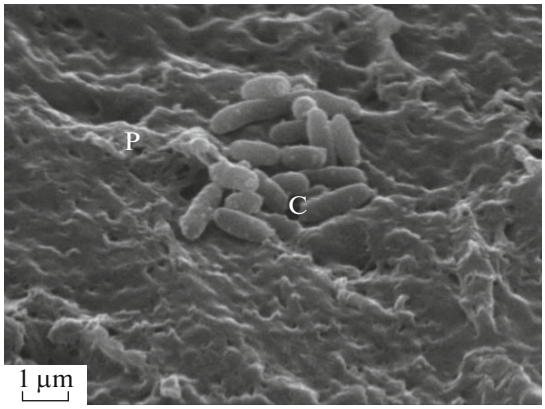

Fig. 2. *P. phosphoreum* immobilized in the matrix of polyvinyl alcohol (PVA) at 10%. The designations are as on Fig. 1. Scanning electron microscopy (SEM). Scale bar, 1 μm.

teristics of the matrix. The structure of the formed gel should affect the bond strength between the cells and the carrier, cell activity, and diffusion parameters of the analyzed compounds. Effect of cryogel concentration on emission activity and bioluminescence stability of the cells was therefore studied. At PVA concentrations from 7 to 14%, rugged elastic structures were formed, in which immobilized cells had similar luminescence characteristics. At carrier concentrations below 7%, loose structures with unstable luminescence were formed.

Electron micrographs of the cells of *Photobacterium phosphoreum* strain 331 KKM MGU immobilized in PVA cryogel are presented on Figs. 1 and 2. When the concentration of the initial PVA solution was 10%, diameter of most of the pores formed during freezing was below 0.5 μm. At 1% concentration of the initial PVA solution, pore size exceeded 2 μm. The number of cells immobilized in 1% PVA granules was therefore significantly higher than in the case of 10% PVA. Differences in cell shape and localization of *P. phosphoreum* in the PVA matrix were observed. In the case of 10% PVA matrix, small colonies were formed (18–20 cells), and the average cell size was 1.5 μm. In the case of 1% PVA matrix, larger colonies developed, which consisted of over 200 of small cells (0.3 μm) embedded in mucous extracellular matrix. Release of bacteria from dense into the incubation medium was rather low (>1%) and occurred during the firs 24 h of incubation, indicating washout from the outer layers. Diffusion of the cells from loose cryogel structures occurred continuously throughout the period of observation.

Bioluminescent activity of immobilized photobacteria. Since bioluminescence intensity and duration vary considerably among photobacterial strains, selection of the strain for immobilization is of importance (Nealson, 1978). The most widespread psychrophilic and mesophilic species were chosen for comparative analysis. Immobilization in PVA cryogel was found to result in increased duration and intensity of luminescence in all studied strains. Data on duration and integral emission activity for free and immobilized cells of bacteria varying in inherent bioluminescence intensity and duration of the luminescence cycle. Under optimal cultivation conditions for each strain, the psychrophilic strains 331 KKM MGU isolated from the White Sea water exhibited the highest values for bioluminescence intensity and duration, while mesophilic *V. harveyi* showed the lowest ones.

It was shown for psychrophilic bacteria (*P. phosphoreum* 331 KKM) that using a complex optimized medium for submerged cultivation as a medium for gel formation enhanced and stabilized emission of bacteria in the carrier (Efremenko et al., 2010). In this case the cryogel is probably enriched with substrates and protectors, this phenomenon probably being common to all photobacteria. Comparative analysis of initial specific luminescence activity and of luminescence duration was carried out for other bacterial species (*V. harveyi*, *V. fischeri*, and *P. phosphoreum*) immobilized into cryogel based on the cultivation medium and 3% NaCl. Specific activity of the granules measured immediately after the restoration of metabolism revealed that in the preparations formed on the cultivation media all three species recovered almost 100% of their luminescence activity after cryogenic immobilization and thawing. Immobilization in the gels formed with 3% NaCl resulted in a considerable (10^2–10^3 times) decrease in emission level. These data are in agreement with the earlier results for *P. phosphoreum* (Efremenko et al., 2010). Dynamics of luminescence of the granules formed in the cultivation medium with immobilized cells of different bacterial strains incubated at 4°C in 3% NaCl is shown on Fig. 3.

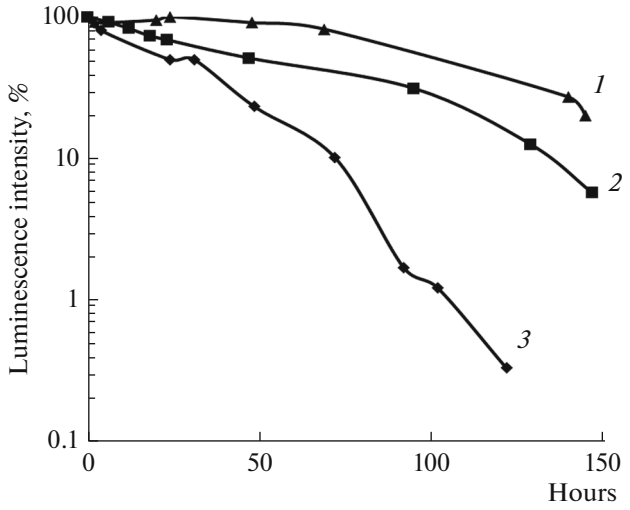

Fig. 3. Light emission dynamics of immobilized cells incubated in 3% NaCl: psychrophilic White Sea *P. phosphoreum* strain 331 KKM MGU (*1*), *P. phosphoreum* 11040 ATCC (*2*), and *V. harveyi* 392 ATCC (*3*). Cell concentration in the granules was 3×10^7 cells/mL.

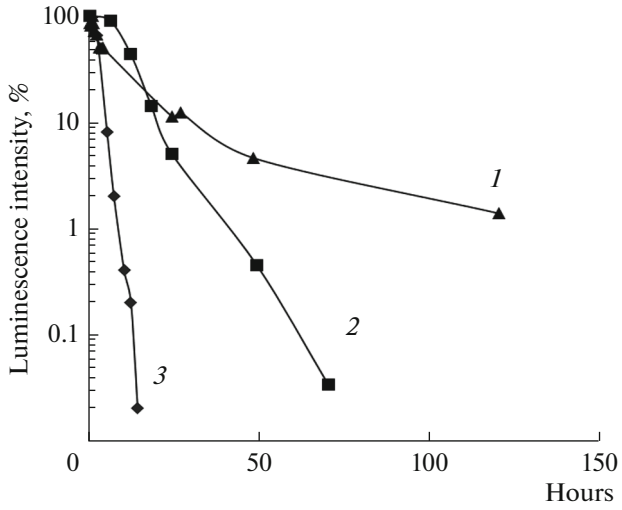

Fig. 4. Dynamics of luminescence fading for free cells of bacteria. The designations are as on Fig. 3. Cell concentration was 10^7 cells/mL. Luminescence intensity was recalculated in per cent.

For comparative analysis of bioluminescence dynamics in free and immobilized cells, a portion of biomass was resuspended in 3% NaCl, and light emission dynamics of free cells was analyzed under the same conditions (Fig. 4).

Oxygen did not limit the bioluminescence activity at the cell concentrations used. Oxygen consumption by the cells of photobacteria varies from 50 to 80 nmol/10^9 cells (Nealson, 1978; Aleskerova et al., 2014). Importantly, for all strains the decay rate was considerably higher for free cells compared to immobilized cells, although the effect of emission stabilization was different in different strains. *V. harveyi* cells exhibited the least stability under storage at 4°C, while the most stable bioluminescence was observed for the psychrophilic strain *P. phosphoreum* 331 (KKM MGU).

Temperature dependences of the luminescence of free and immobilized cells. The cells of photobacteria are known to be highly sensitive to temperature, with the range for growth and light emission from 4 to 30°C. The highest luminescence of different strains occurred at 15–28°C (Nealson, 1978). At lower temperatures cell growth was suppressed and the bioluminescence activity decreased slightly. The temperature factor may have a crucial effect on bacterial activity in the case of long-term experiments. Stabilization of light emission by immobilized cells was probably to a certain degree associated with the changed temperature stability of bioluminescence. Differences in the temperature profiles of light emission by immobilized cells compared to free ones was to be expected. Analysis of temperature dependence of free and immobilized cells of psychrophilic bacteria *P. phosphoreum* KKM 331 MGU was carried out previously (Efremenko et al., 2014). Broader temperature dependence of light emission and an insignificant shift of its maximum for immobilized cells to higher temperatures (Efremenko et al., 2014). Analysis of the temperature dependence of other species (*P. phosphoreum* ATCC 11040, *V. harveyi* 392) revealed similar profiles of the effect of temperature on light emission by free and immobilized cells. The temperature dependence of *V. harveyi* free and immobilized cells is shown on Fig. 5. An insignificant stabilization of light emission was observed at elevated temperatures.

Effect of the composition of the incubation medium on the luminescence of immobilized preparations. The issue of the reasons for bioluminescence fading in growing cultures and in isolated and especially in immobilized cells remains open. Limitation in vitro by the luciferase aldehyde cosubstrate and/or reducing equivalents supporting the FMN · H_2 pool may be responsible for bioluminescence fading in vitro.

Experiments with different media revealed the kinetic profile of light emission in a carrier depended on the buffer capacity and initial pH of the incubation medium. Comparative analysis of the fading rate in a buffer medium and in a simple salt solution revealed that bioluminescence duration in the solution was considerably lower than in the buffer medium (Fig. 6). Moreover, both initial intensity and stability of light emission were higher in alkaline media than in neutral ones. This finding agrees with the data on pH dependence for the bioluminescence of free bacteria (Kuts and Ismailov, 2009). After fading in 3% NaCl, emission activity of the cells was completely restored by transfer into an alkaline medium (0.1 M carbonate buffer, pH 8.5).

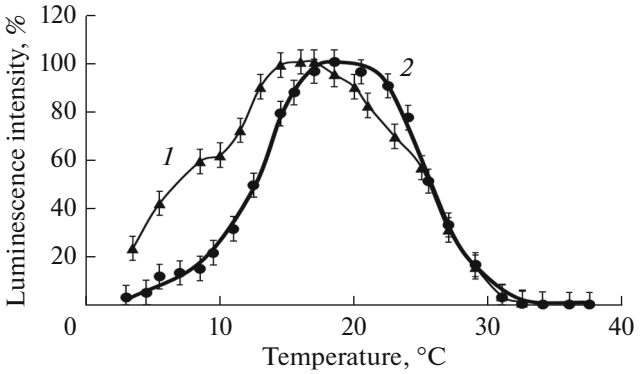

Fig. 5. Temperature dependence of the luminescence of free (*1*) and immobilized (*2*) *V. harveyi* cells in 2% NaCl.

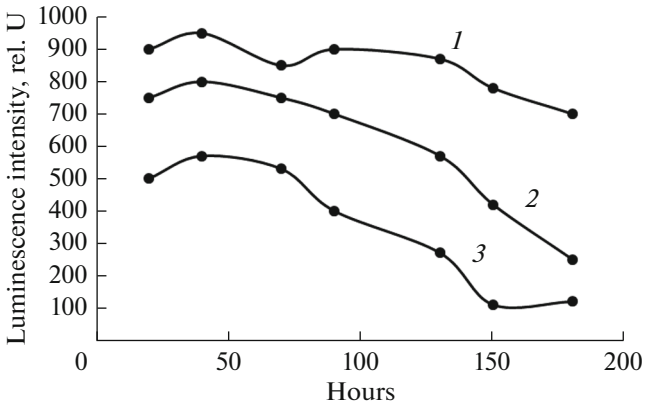

Fig. 6. Luminescence kinetics of immobilized cells of *P. phosphoreum* 331 KKM MGU in different incubation media: 0.1 M NaP carbonate + 2% NaCl, pH 8.5 (*1*); 0.1 M NaP carbonate + 2% NaCl, pH 7.6 (*2*); and 3% NaCl (*3*).

DISCUSSION

As was noted above (Chun et al., 1996; Park et al., 2005), immobilization may result in enhanced activity and stability of the cellular biological processes. The stress effects of the immobilization procedure, as well as the state of the cells in a matrix may, however, affect kinetic and emission characteristics of bacteria. Residual specific activity of the cells after immobilization was not discussed in detail in most works. For the cells immobilized in agar, agarose, or alginate, suppression of the luminescence activity is associated with the immobilization procedure, primarily due to the sensitivity of the cells to relatively high temperatures of gel formation (up to 30–50°C for different carriers) (Chun et al., 1996). Partial replacement of bivalent ions in the gel by Na^+ ions, which occurs during prolonged incubation of the preparations in salt solutions, may also destabilize the matrix.

The results obtained in the present work make it possible to suggest that selection of a relevant strain is the main factor affecting production of immobilized preparations with intense and stable light emission. Among the known photobacteria, *P. phosphoreum*, with the most intense (10^5 quanta/(s cell)) and prolonged (over 100 h in submerged cultures) light emission (Kuts and Ismailov, 2009), satisfy these requirements to the highest degree.

The structure of the formed cryogel plays an important role, since it should prevent bacterial release from the matrix, while not limiting diffusion of the analyzed compounds. Optimal cryogel concentrations were found to be from 10 to 14%.

An important observation concerning the media used as a basis for gel formation should be stressed. Using the optimized medium for submerged cultivation of photobacteria provided for intense, long-term light emission. Rapid fading of bioluminescence was reported for the cells of *V. fischeri* and of the genetically engineered *P. putida* strain bearing the *lux* operon of *P. luminescence* immobilized in PVA cryogel (Philp et al., 2003). Glycerol was added to the preparations to increase stability of emission. The procedure also included the stage of activation by incubation in special media. High noise level and rapid bioluminescent fading were noteworthy and resulted probably from low specific activity, although this parameter was not reported. The gels were formed in NaCl solutions, which was the possible cause of unstable light emission. Our results indicate that gel formation in this medium may result in decreased activity and stability of light emission.

The temperature dependences for free and immobilized cells indicate a certain stabilization of their activity by PVA cryogel, as was evidenced by a shift of the bioluminescence maximum to higher temperatures. Thus, the stress action of immobilization had no significant effect on stability of the cells in the carrier.

The metabolic potential of the cell, providing for the reduced state of the luciferase flavin substrate, is of special importance. Deposition by electron donors and substrates of the luciferase reaction in the cells of photobacteria is carried out due to sequential NADH:FMN oxidoreductase and luciferase reactions:

$$NADH + H^+ + FMN^- \rightarrow NAD^+ + FMNH_2$$
(oxidoreductase),

$$FMNH_2 + RCHO + O^{2-}$$
$$\rightarrow FMN + RCOOH + H_2O + h\nu \text{ (luciferase)}.$$

Due to rapid nonenzymatic $FMNH_2$ oxidation by oxygen of the air and to the competition of other electron acceptors, reduction rate of the flavin substrate is the main limiting stage of the luciferase reaction. In a coupled system of electron transfer

$$\text{NAD-dependent dehydrogenases}$$
$$\rightarrow NADH + H^+ \rightarrow FMNH_2,$$

Integral photon yield and light emission duration for free and PVA-immobilized bacteria *P. phosphoreum* ATCC 11040, *P. phosphoreum* KKM MGU 331, *V. harveyi* 292 MAV, *V. fischeri* KKM MGU 6 in the course of incubation in 0.1 M Na-phosphate buffer with 2% NaCl, pH 7.6 at 4°C. The concentration of immobilized cells was $1–5 \times 10^7$ cells/granule, the concentration of free cells was 3×10^7 cells/mL

Strains	Immobilized cells		Free cells	
	integral photon yield Q, quanta/cell	luminescence duration, days	integral photon yield Q, quanta/cell	luminescence duration, days
P. phosphoreum ATCC 11040	$1–5 \times 10^7$	14	10^6	3
P. phosphoreum KKM MGU 331	$1–5 \times 10^9$	42	10^8	14
V. harveyi 292 MAV	$1–5 \times 10^6$	3	10^5	1
V. fischeri KKM MGU 6	$1–5 \times 10^6$	5	10^5	2

the rate of NAD^+ reduction and therefore of FMN reduction increases in alkaline media. This may be the reason for enhanced activity and stability of light emission at pH 8.0–9.0.

The aliphatic aldehyde, the cosubstrate of the luciferase reaction, is formed in the reaction of fatty acid reduction by the fatty acid reductase:

$$RCOOH + ATP + NADPH^- \rightarrow RCHO + AMP + PPi + NADP^+.$$

The absolute values of photon yield and ATP content indicate that the substrates within the granules formed in the cultivation medium may be sufficient for flavin reduction and aldehyde generation. It was shown previously (Aleskerova et al., 2014) that the ATP pool in immobilized *P. phosphoreum* cells incubated at 4°C remained almost stable ($0.5–2.0 \times 10^{18}$ mol/cell) throughout the incubation of over 100 h in 3% NaCl. The present work provided similar results for *V. harveyi*. ATP content in these bacteria remained at $0.1–0.5 \times 10^{-18}$ mol/cell during 24-h incubation. Our results indicate that no leakage of the energy donors maintaining the reduced state of flavin and the precursors of aliphatic aldehyde biosynthesis occurs, which has been hypothesized previously (Makiguchi et al., 1980). Thus, no limitation of emissive activity of the cells by luciferase cofactors and substrates occurred in cryogels formed in the cultivation medium.

The data on fading kinetics in different media (Fig. 6) confirm that pH shift caused by acidic metabolic products is one of the major factors responsible for bioluminescence fading, as was previously suggested for *P. phosphoreum* (Alenina et al., 2012). Alkaline conditions and high buffer capacity of the incubation media are therefore required for the maximally prolonged light emission.

The results on investigation of the most crucial elements controlling light emission activity in PVA-immobilized photobacteria indicate that specific emission characteristics of the biological object are of utmost importance for production of highly stable luminescent biosensors. Composition of the medium of gel formation, as well as the buffer capacity and pH of the incubation medium, are also of considerable importance.

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