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BIOREPORTER *PSEUDOMONAS* *FLUORESCENS* HK44 IMMOBILIZED IN A SILICA MATRIX

The bioluminescent bioreporter Pseudomonas fluorescens HK44, the whole cell bacterial biosensor that responds to naphthalene and its metabolites via the production of visible light, was immobilized into a silica matrix by the sol-gel technique. The bioluminescence intensities were measured in the maximum of the bioluminescence band at $\lambda = 500$ nm. The immobilized cells ($>10^5$ cells per g silica matrix) produced light after induction by salicylate (conc. $>10^{-4}$ g/l), naphthalene and aminobenzoic acid. The bioluminescence intensities induced by 2,3-dihydroxynaphthalene, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were comparable to a negative control. The cells in the silica layers on glass slides produced light in response to the presence of an inductor at least 8 months after immobilization, and >50 induction cycles. The results showed that these test slides could be used as assays for the multiple determination of water pollution.

Bioluminescent bioreporters were originally constructed as whole-cell bacterial biosensors that responded to specific chemical or physical agents in their environment via the production of visible light [1,2]. These organisms utilized a genetic construct consisting of the lux gene cassette derived from the marine bacterium *Vibrio fischeri*. The lux cassette consists of five genes, luxA, B, C, D, and E. The luciferase genes (*luxAB*) encoded for the proteins responsible for generating bioluminescence, while the reductase (*luxC*), transferase (*luxD*) and syntethase (*luxE*) genes encoded for proteins involved in the production of the aldehyde substrate required in the bioluminescent reaction [3,4]. Coupling of the lux cassette to an inducible promoter gene generates a bioreporter capable of generating visible light in a target specific manner, with no requirement for the extraneous addition of substrate. Thus these bioassays can be performed repetitively in real-time and on-line. Bioreporters are primarily employed as environmental sensors, where their real-world application requires an encapsulation matrix that is strong enough to endure the rigors of the outside environment, yet resilient enough to viably maintain the fragile cells.

Pseudomonas fluorescens HK44 harbours the pUTK21 plasmid, derived from NAH7 plasmid, which codes genes for the naphthalene degradation pathway divided into two operons. Both operons are positively inducible by salicylate, therefore little of their activity is present constitutively and a huge increase of activity is observed after induction. *Vibrio fischeri luxCDABE* gene cassette coding bioluminescence was inserted into the

nahG gene of the salicylate operon, thus gaining the inducibility by salicylate or naphthalene [5–10].

The formation of inorganic and organic/inorganic materials by the sol-gel method has been used to immobilize enzymes, nucleic acids, antibodies, organic compounds, living cells, and dead cells [12]. An intensive research effort in this field uncovered some benefits of biomaterials prepared by their immobilization into inorganic matrices that could not be achieved by their immobilization on the surface of inorganic materials or by their encapsulation into organic prepolymers. The most important properties of the inorganic matrices are biocompatibility, transparency, and chemical, thermal and dimensional stability. Furthermore, the porosity of the gel permits nutrients to reach the organism and by-products to escape. These materials are thus superior to organic matrices in a great number of applications, such as in medicine [13], the construction of optical sensors [14], enzyme catalysis at elevated temperatures [15], in non-aqueous solvents [16] and in the encapsulation of cells for organic pollutant degradation [11].

Conventional sol-gel procedures involve extremes of pH and high concentrations of alcohol, both of which are usually detrimental to the stability of biomaterials. To overcome these obstacles, modified techniques were developed [17,18]. The biomaterial to be encapsulated is added to the sol after partial hydrolysis of the precursor. As the degree of cross-linking due to polycondensation increases, the gel becomes viscous and solidifies. The process continues during aging, and a porous matrix is formed around the biomaterial trapping it inside. In contrast to covalent attachment or surface adsorption, this process produces a bulk material in which the biomaterial is captured even though it has not reacted with the matrix or by some other way attached to it.

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In this work we immobilized the bioluminescent bioreporter *Pseudomonas fluorescens* HK44 into thick silica layers prepared from prepolymerized tetramethoxysilane. The response time, the effect of the cell concentration in the layer and the intensity of bioluminescence induced by different concentrations of salicylate and naphthalene, as well as other possible inducers were followed.

EXPERIMENTAL

Microorganisms and media

P. fluorescens HK44 were cultivated in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) with tetracycline (50 mg/l). Bioluminescence was induced in YEPS medium (yeast extract 0.2 g/l, ammonium nitrate 0.2 g/l, sodium succinate 2.7 g/l, polypeptone 1 g/l). Phosphate buffer (0.066 mol/l, pH 7) was used for the extinction of bioluminescence and during storage in the fridge.

Cells were grown overnight in 250 ml Erlenmeyer flasks with 50 ml of LB medium with tetracycline at 25°C; 10 ml of suspension were then inoculated into a 250 ml Erlenmeyer flask with 100 ml of LB with tetracycline and grown to OD 0.8. The cells were then centrifuged (5 min., 5°C, 9000 min⁻¹), resuspended in YEPS medium, centrifuged again (5 min., 5°C, 9000 min⁻¹) and resuspended in 2 ml LB without tetracycline. The suspension was diluted by LB medium without tetracycline to the desired cell concentration (8x10²–8x10⁴–8x10⁶–8x10⁸–8x10⁹ per gram of final material).

Immobilization

Microscopy glass slides (76x26 mm, aprox. 5 g) were first wiped with ethanol, toluene and acetone, then bathed overnight in 1M NaOH, washed with distilled and deionized water, treated for 20 minutes in an ultrasonic bath in deionized water and, finally, dried for 2 hours at 120°C.

Tetramethoxysilane (TMOS, Fluka product No. 87682) was stirred with distilled water and HCl in the molar ratio TMOS/H₂O/HCl = 1:5:10⁻² to form a clear solution and left to prepolymerize for 48 hours at 4°C.

Prepolymerised TMOS (0.5 ml) was mixed with 0.5 ml of 0.05 M NaOH. The cell suspension was then added (2 ml), the mixture was vortexed and poured on the microscopy glass slides in a Petri dish. After 5 minutes of drying at 25°C the glass slides were washed with phosphate buffer (10 ml) and then stored overnight in phosphate buffer at 4°C.

Luminescence induction and measurement

Bioluminescence was induced in the YEPS medium by addition of the tested compounds. Sodium salicylate and naphthalene were used as known

inducers. Naphthalene (Lachema Praha) was added at concentrations below saturation, approx. 31 mg/l (Heitzer A. et al., 1992), sodium salicylate (Lachema No. 30078 0103) was added to the final concentration 0.5 g/l and lower. The concentration series 5x10⁻¹ – 5x10⁻² – 5x10⁻³ – 5x10⁻⁴ – 5x10⁻⁵ g/l of sodium salicylate was used to determine the minimal inducing concentration. 2-Aminobenzoic acid (Merck No. 820112), 3-hydroxybenzoic (Aldrich No. 09227CS-442) acid, 4-hydroxybenzoic (Aldrich No. S05151-232) acid 0.5 g/l and 2,3-dihydroxynaphthalene (Aldrich No. 17407BO-322), applied as saturated solutions, were also tested. Layers in the YEPS medium with inducer were incubated at 25°C. Incubation in the phosphate buffer was used to extinct bioluminescence.

A layer in YEPS was used as a negative control. A layer in YEPS + 0.5 g/l of sodium salicylate was used as a positive control.

The bioluminescence of the layers in the Petri dish was measured by a F-4500 HITACHI fluorometer in the wavelength range 400–600 nm. The photo multiplier noise was eliminated by subtracting the luminescence intensity at $\lambda = 420$ nm (minimum of the bioluminescence band) from the luminescence intensity at $\lambda = 500$ nm (maximum of the bioluminescence band).

RESULTS AND DISCUSSION

Figure 1 shows layers of immobilized cells with concentrations of 2x10⁸, 1x10⁹, 4x10⁹ and 1.5x10¹⁰ cells/g. The weight of the layers was 2±0,2 g, the thickness was 1±0,1 mm.

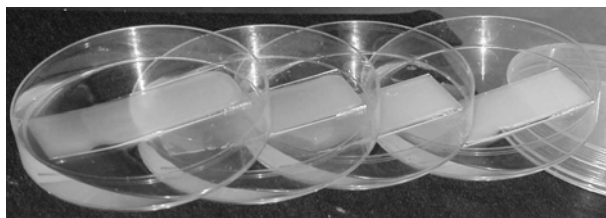


Figure 1. Layers with cell concentrations 2x10⁸, 1x10⁹, 4x10⁹, 1.5x10¹⁰ cells/g

After 8 months and more than 50 induction cycles, the layers with cell concentrations 10⁵–10⁷ were mechanically undamaged, in contrast to layers with cell concentrations >10⁷ that were eroded (losing ~20–50% of their weight). Bioluminescence of the immobilized cells remained unchanged regardless of the cell concentration and erosion of the layer.

The time dependence of the bioluminescence induced by sodium salicylate (0.5 g/l) is shown in Figure 2. The curve resembles the growth-curve, which coincides with the induction of the *lux* genes.

The influence of the cell concentration in the layer on the intensity of bioluminescence after 3 hours of

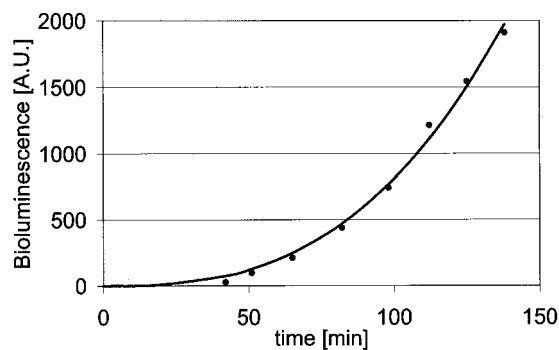


Figure 2. Bioluminescence intensity of the layer with 4×10^9 cells/g induced by YEPS + 0.5 g/l salicylate.

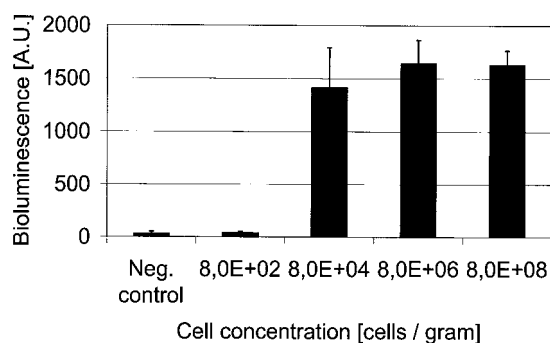


Figure 3. Influence of the cell concentration on the bioluminescence response on YEPS + 0.5 g/l salicylate after 3 hours of incubation.

incubation in YEPS + 0.5 g/l sodium salicylate is shown in Figure 3. In comparison to the layers with cell concentrations of $8 \cdot 10^4$ cells/g – $8 \cdot 10^8$ cells/g, the intensity of bioluminescence of the layers with the cell concentration $8 \cdot 10^2$ cells/g was only 1x – 2x higher than that of the control samples, which is at the level of the photo multiplier noise.

The bioluminescence of the layers did not increase proportionally to the cell concentration, probably due to the slow diffusion in the thick silica layer. The light was also more attenuated in layers with higher cell density.

Bioluminescence might also be theoretically induced by compounds that are structurally similar to salicylate such as 3-hydroxybenzoic acid, 4-hydroxybenzoic acid and 2-aminobenzoic acid or by compounds that can be metabolized to salicylate through the upper naphthalene-degradation pathway such as 2,3-dihydroxynaphthalene. From the tested compounds only 2-aminobenzoic acid is inducer comparable to the salicylate (see Figure 4).

Figure 5 shows that the minimal tested concentration inducing bioluminescence was 0.005 g/l and the maximal tested concentration not inducing bioluminescence was 0.0005 g/l. Based on the data obtained by Heitzer A. et al. [10], we can expect that the detection limit might be lower in a continuous system and after optimization of the cell concentration.

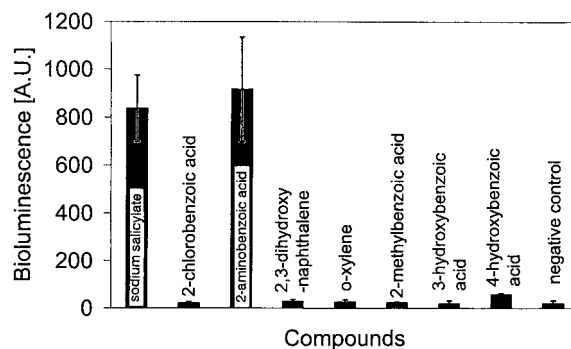


Figure 4. Response of the layers with the cell concentration 8×10^6 to possible inducers of bioluminescence after 3 hours of incubation in YEPS + 0.5 g/l inducer (2,3-dihydroxynaphthalene in saturated solution).

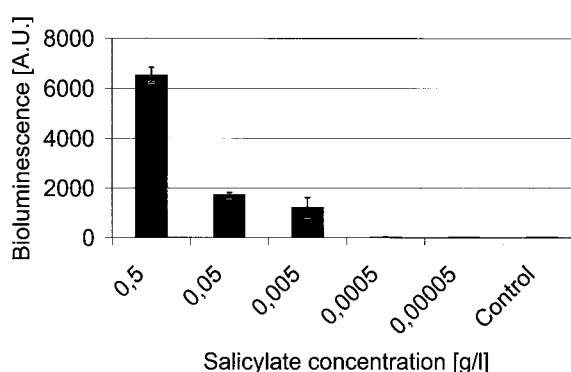


Figure 5. Dependence of bioluminescence on the salicylate the concentration. Layers with cell concentration $8 \cdot 10^8$ were immersed in YEPS medium at 25°C. Bioluminescence was measured after 3 hours of incubation.

CONCLUSION

Thick silica layers, prepared from prepolymerized tetramethoxysilane with immobilized *Pseudomonas fluorescens* HK44, showed a detection limit of 0.005 g/l sodium salicylate and at least 8 month stability without decrease of the detection parameters. The intensity of bioluminescence was not substantially changed by variation of the cell concentrations in the range $\sim 10^5$ – 10^9 cells/gbiocomposite. Strong luminescence was induced selectively by salicylate, naphthalene and 2-aminobenzoic acids. These properties together with the simplicity of the layer preparation proved that layers on glass slides can be used as a simple and selective assay of environmental pollution.

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IZVOD

IMOBILIZACIJA ĆELIJA BIOREPORTERA PSEUDOMONAS FLUORESCENS HK44 U SILIKATNOJ MATRICI

(Naučni rad)

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Ćelije bioluminiscentnog bioreportera, *Pseudomonas fluorescens* HK44, bakterijskog biosenzora koji reaguje na prisustvo naftalina i njegovih derivata odavanjem vidljive svetlosti, imobilisane su u silikatnoj matrici primenom sol-gel tehnike. Intenziteti bioluminiscencije su mereni na maksimalnoj vrednosti talasne dužine za bioluminiscenciju, $\lambda = 500$ nm. Imobilisane ćelije pri koncentraciji od $>10^5$ ćelija po g silikatne matrice su proizvele svetlost nakon uvođenja salicilata (konc. $>10^{-4}$ g/l), naftalina i aminobenzojeve kiseline. Intenziteti bioluminiscencije indukovani pomoću 2,3-dihidroksinaftalina, 3-hidroksibenzojeve kiseline i 4-hidroksibenzojeve kiseline bili su zanemarljivi, reda veličine negativne kontrole. Ćelije imobilisane u silikatnim slojevima na staklu su proizvele svetlost kao odgovor na prisustvo induktora u dugom vremenskom periodu, najmanje 8 meseci nakon imobilizacije, odnosno u preko 50 indukcionih ciklusa. Rezultati ukazuju da ovi test senzori sa imobilisanim ćelijama mogu biti korišćeni za višestruku detekciju zagađenja vode.

Ključne reči: Biolumiscentni bioreporter • Optički senzor • Detektovanje naftalina • Detektovanje salicilata •

Key words: Bioluminescence bioreporter • Optical sensor • Naphthalene detection • Salicylate detection •