Encapsulation of bacteria for construction of whole-cell optical biosensors.

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Introduction

In old industrial factories a localization of polluted zones requires many analyses. A screening with whole cell biosensors is a cheap semiquantitative detection of the most contaminated spots, which avoids using of laborious analytical method and expensive instrumentation. Encapsulation matrices provide protection and can contain nutrients and hydration to help sustain cells in a physiological state necessary for sensing. Among different types of encapsulation matrices, which have been used for the entrapment of nutrients and viable micro-organisms in thin films or beads, silica gel is chemically inert and optically transparent.
In this paper we present two whole cell optical screening assays with bacteria immobilized into silica gel layers: 1) detection of naphthalene, salicylate and their analogs via measuring of bioluminescence produced by bioluminescent bioreporter *Pseudomonas fluorescens* HK44
2) semiquantitative detection of polychlorinated biphenyls (PCBs) with 3 chlorine atoms with *Pseudomonas species 2* which degrade these PCBs, in cometabolization with biphenyl, forming stable yellow *meta* ring-fission products 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDA) that are accumulated in medium. Light absorbance of a medium at λ= 400 nm is proportional to PCBs concentration.

Material and Methods

Microorganisms and media
Pseudomonas fluorescens HK44 is harboring the pUTK21 plasmid, derived from NAH7 plasmid, which codes genes for naphthalene degradation pathway divided into two operons. Both operons are positively inducible by salicylate, therefore little of their activity is present constitutively and huge increase of activity is observed after induction. *Vibrio fischeri luxCDABE* gene cassette coding bioluminescence was inserted into *nahG* gene of the salicylate operone, thus gaining the inducibility by salicylate or naphthalene (Heitzer et al.1992). *Pseudomonas fluorescens* HK44 were cultivated in LB medium with tetracycline overnight.
Pseudomonas species 2, isolated from PCB contaminated soils in the Czech Republic (Pazlarova at al. 1997), were incubated in mineral medium with biphenyl (3 gl^{-1}) as sole carbon source for one day.
Pre-polymerization of tetramethoxysilan (TMOS)
TMOS, Fluka product No. 87682, was stirred with distilled water and HCl in molar ratio TMOS/H_2O/HCl = 1:5:10^{-2} to form a clear solution and left to pre-polymerize for 48 hours at 4°C.
Whole cell biosensor of naphthalene and salicylate.
Pre-polymerized TMOS was mixed with 0.05 M NaOH (0.1 ml) and with HK44 cell suspension in LB medium (0.4 ml, concentration of 10^{-7} cells ml^{-1}). The mixture was poured into Petri dishes Ø 3.5 cm to form silica film thickness of 1 mm. Bioluminescence of HK44 layers was monitored using Berthold FB-12 luminometer.
Whole cell biosensor of PCB.
Pre-polymerized TMOS (0.15 ml) containing 0.1 gl^{-1} of biphenyl was mixed with 0.05 M NaOH (0.15 ml) and with *Pseudomonas species 2* cell suspension in mineral medium (0.5 ml,

concentration of $6x10^8$cells ml^{-1}). The mixture was poured into Petri dishes Ø 3.5 cm. The film containing $4x10^8$ cells g_{gel}^{-1}, thickness of 1.3 mm and weight 0.75 g (±1%) gelled within 5 minutes at 25°C. The gel was washed with buffer and stored in fridge overnight (10°C). Mineral medium (2 ml) was added together with tested contaminants: commercial mixture of PCBs, Delor103 (D103), which contains 45% of congeners with 3 chlorine atoms, at concentrations of 1 to 20 mgl^{-1}, individual PCB congeners (10 mgl^{-1}), PAHs, naphthalenes, solvents or dibenzofuran (2.5 gl^{-1}). Real PCB contaminated soil was added as methanol extract (2.5 µL). Seven replicates of each sample were used for the statistical interpretation. Petri dishes were incubated for 3 hours at 30°C and were stored at 10°C between experiments. After incubation, 0.6 ml of media was drawn off and the absorption spectra were measured by UV-VIS spectrophotometer HP-8452 in 3 cm (optical length) glass cell. The absorbance values of yellow HOPDA products were obtained by subtraction of the reference absorbance at λ = 600 nm and HOPDA absorption maxima at λ = 398 nm.

Results and Discussion

Silica matrices are relatively inexpensive to synthesize and have several desirable properties including chemical inertness, optical transparency, enhanced thermo stability, biocompatibility, and resistance to microbial attack. Both strains were immobilized into layers on the bottom of small Petri dishes (Fig.1). Cells suffer by immobilization stress due to evolution of methanol in polycondensation reactions and shrinkage during gellation. The cell viability was restored after one day rest in fridge (Fig . 2).

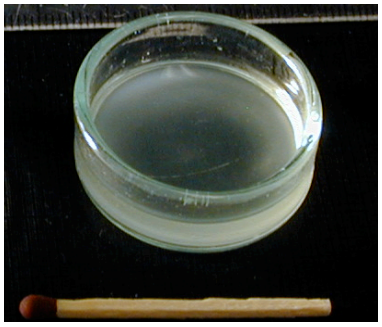


Fig 1.Whole cell sensor = Petri dish with immobilized bacteria into silica gel.

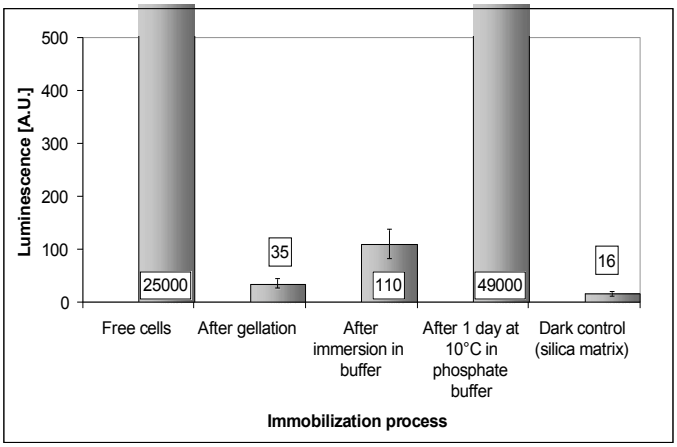


Fig 2. The influence of immobilization process on background luminescence. (Silica matrix 2% Si, 10^8 cell HK44, 25°C).

After immobilization, HK44 performed higher luminescence in first induction as compared to free cells and sometimes, accidentally, they produced light without induction. Small differences in response time of free and entrapped cells (Fig. 3) together with high induced luminescence within 15 induction cycles (Fig. 4) showed that immobilization into ~1 mm thin films cut down influences of mass transport limitations and gel aging, which were observed after entrapment into thicker pieces (Branyik et al. 2000). Increasing of silica content from 2 to 5 % made the layers more durable but luminescence maxima were decreased to one half. Alginate added into prepolymerized silica caused escaping of cells from silica/alginate layers (Kuncová et al. 2004). Limits of detection with free and silica immobilized cells have been identical 0.05 $mg_{naphtalene}l^{-1}$ and 0.5 $mg_{salicylate}l^{-1}$, response time 6 hours, storage stability > 3 month. Bioluminescence of cells HK44 was induced also by some substituted naphtalenes and salicylates (Trögl et al. 2005, 2007). Detection potential

of whole cell sensor was demonstrated by induction of luminescence in presence of contaminated soil, taken from area of old industrial burden, which was polluted with polyaromatic hydrocarbons 1850 $mg_{PAH}kg^{-1}$.

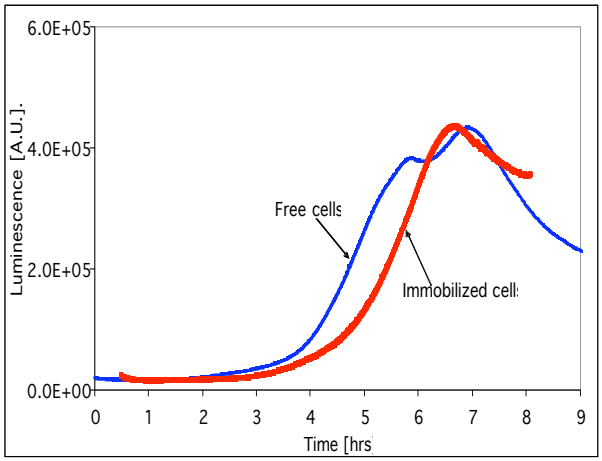


Fig. 3 Kinetics of bioluminescence. (10^8 cells g_{gel}^{-1}, film thickness 2 mm, silica gel 2% Si). Inducer: naphthalene in YEPS (2 ml).

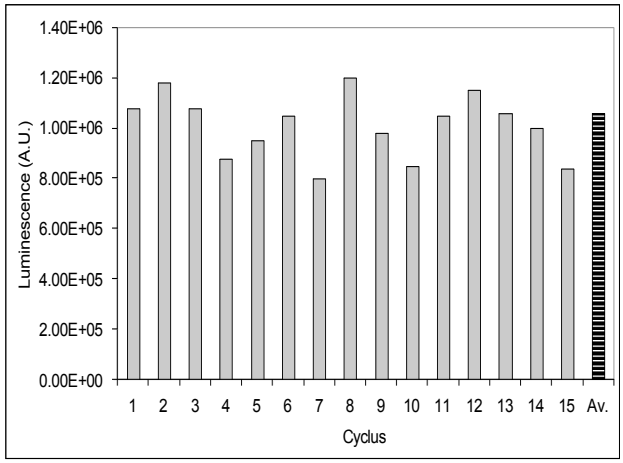
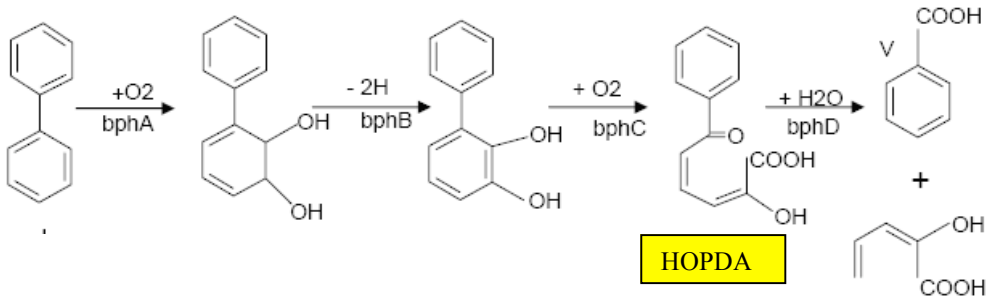


Fig. 4 Repeatability and reproducibility of bioluminescence induction (10^8 cells g_{gel}^{-1}, film thickness 1 mm, silica gel 2% Si) Inducer: naphthalene in YEPS (2 ml).

In whole cell sensor with bioluminescence bioreporter HK4 silica gel serves as inert matrix and might be replaced by another suitable polymer as alginate or chitosan. In case of PCB sensor with *Pseudomonas species 2*, biphenyl was dissolved in tetramethoxysilane so necessary cosubstrate of PCB degradation was uniformly distributed in silica matrix. This improved reproducibility of PCB detection and cohesiveness of silica film. PCBs are co-metabolically transformed to chlorobenzoic acids by aerobic bacteria through the biphenyl catabolic pathway. During the third step of the so called upper PCB metabolic pathway the yellow *meta* ring-fission product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) is formed:



Chlorine in the *ortho* position on one of the PCB ring and chlorine in *para* position on the second ring is the chlorination pattern needed for the production and accumulation of yellow *meta* ring-fission product (Seeger et al. 1999). On Fig. 5 are shown reproducibility and repeatability of production of yellow intermediates after addition of D103 to Petri dish with the silica film with entrapped *Pseudomonas species 2*. Semiquantative detections were interfered with toluene, dibenzofuran, xylene, naphthalene and 1-methylnaphtalene (Fig. 6), in contrast to phenantrene, antracene and pyrene which did not influence PCB detection (Gavlasova et al. 2006). The detection limit of whole cell PCB sensor was 1.2 $mg_{D103}l^{-1}$ and 0.2 $mg_{2,4,4'\text{-chlorobiphenyl}}l^{-1}$, response time 3 hours, reproducibility of PCB determination ± 10%, reusability 3 times and ≥ 4 weeks storage stability was demonstrated. D103 was detected in samples of soil contaminated with 80-83 $mg_{2,4,4'\text{-chlorobiphenyl}}$ kg^{-1} which were taken from landfill area. PCBs were semiquantitatively determined after soil co-entrapment into silica gel and also after addition of methanol extract into medium in Petri dish.

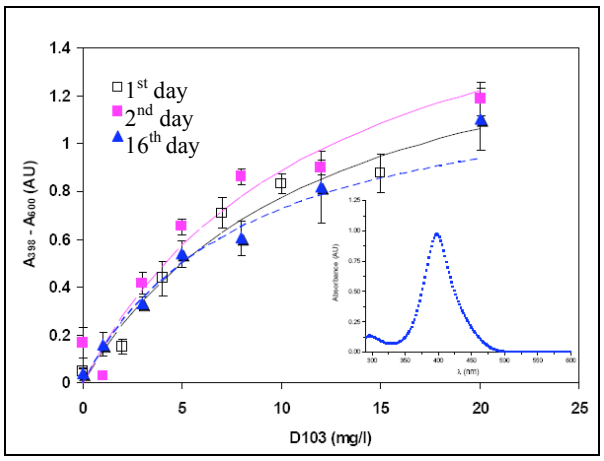


Fig. 5. Evolution of yellow intermediates after three hours incubation as a function of D103 concentration. 1, 2 and 16 days after cell entrapment. Insert: light absorption spectrum of a medium with 15 $mg_{D103}l^{-1}$.

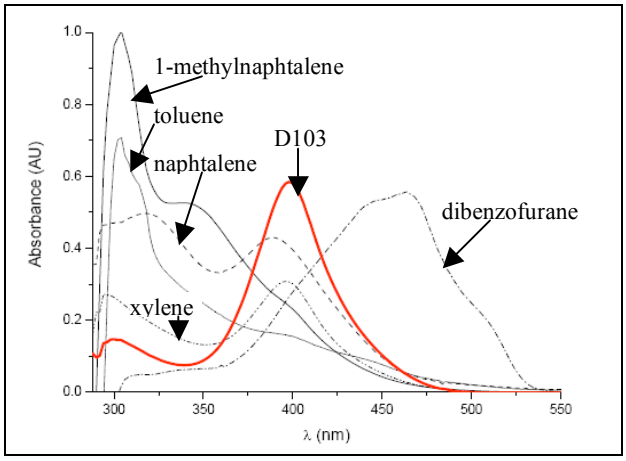


Fig. 6. Light absoption spectra of the medium in presence of D103 and compounds interfering whole cell optical detection (after 3 hours cultivation).

Conclusions

In this work we demonstrated entrapment of two living bacterial strains into silica gel and application of prepared films in optical detection of pollutants. A modification of sol-gel process, as an alcohol free route (Ferrer et al. 2002) or addition of organic polymers (Livage et al. 2006), to protect cells against harsh conditions during sol-gel entrapment was not used. In water, detection limits of both assays were in order of tenth milligrams per liter. Evaluation of applicability of whole cell sensors for detection of contaminants in soils needs more experiments.

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