Soil biosensor for the detection of PAH toxicity using an immobilized recombinant bacterium and a biosurfactant

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Abstract

A biosensor for detecting the toxicity of polycyclic aromatic hydrocarbons (PAHs) contaminated soil has been successfully constructed using an immobilized recombinant bioluminescent bacterium, GC2 (lac::luxCDABE), which constitutively produces bioluminescence. The biosurfactant, rhamnolipids, was used to extract a model PAH, phenanthrene, and was found to enhance the bioavailability of phenanthrene via an increase in its rate of mass transfer from sorbed soil to the aqueous phase. The monitoring of phenanthrene toxicity was achieved through the measurement of the decrease in bioluminescence when a sample extracted with the biosurfactant was injected into the minibioreactor. The concentrations of phenanthrene in the aqueous phase were found to correlate well with the corresponding toxicity data obtained by using this toxicity biosensor. In addition, it was also found that the addition of glass beads to the agar media enhanced the stability of the immobilized cells. This biosensor system using a biosurfactant may be applied as an in-situ biosensor to detect the toxicity of hydrophobic contaminants in soils and for performance evaluation of PAH degradation in soils. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Soil biosensor; Immobilized recombinant bioluminescent bacterium; Bioavailability of PAH; Biosurfactants; Phenanthrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of solid phase organic chemicals containing two or more fused benzene rings (Zhang et al., 1997). The occurrence of PAHs in soils, sediments, aerosols, water, animals and plants is of increasing environmental concern because PAHs may exhibit mutagenic and carcinogenic effects (Henner et al., 1996). These compounds may be released into the environment through the disposal of coal tar and other coal processing wastes, petroleum sludge, asphalt, creosote, and other wood preservative wastes, chemical waste, and soot (Laha and Luthy, 1991). PAHs and their degradation products in soils endanger human health but also soil and aquatic ecosystems by directly affecting soil biota or, after runoff or percolation, water or groundwater biota. Hazard and risk assessment of polluted soil is usually performed by means of physical and chemical measurements, but chemical analysis may not be enough due to the fact that it is restricted to a limited list of defined and extractable compounds (Bispo et al., 1999). It is also difficult to analyze chemical compounds with a high heterogeneity of toxic properties since a thorough analysis includes complicated procedures. In addition, no information about toxicity of the samples can be given from the physico-chemical analysis.

Therefore, alternative methods for the detection of toxicity are needed, such as biological tests, which consist of exposing biological organisms to polluted materials. However, the detection of PAHs toxicity in the environment is known to be restricted due to their low solubility and sorption to solid surfaces. The hydrophobic nature of these contaminants results in their partitioning onto the soil matrix, limiting their bioavailability (Guha and Jaffe, 1996a). In addition, the sorbed and solid phase PAHs are not directly available to microorganisms, due to the limited rate of mass
transfer of PAHs from the sorbed and the solid phase to the aqueous phase (Zhang et al., 1997; Guha and Jaffe, 1996b). Furthermore, in-situ detection of the toxicity of PAHs contaminated soil has not been successfully conducted because of the absence of appropriate biosensing cells as well as tools and techniques.

Surfactants can enhance the rate of mass transfer from the solid and sorbed phases by increasing the rates of dissolution and desorption of PAHs (Zhang et al., 1997). Specifically, microbiologically produced surfactants, or biosurfactants, offer the advantage of being potentially less toxic and more biodegradable than some synthetic surfactants (Torrens et al., 1998). The surfactant concentration at which its monomers begin to assemble in ordered colloidal aggregates is termed the critical micelle concentration (CMC). Solubilization is attributed to incorporation of the hydrophobic PAHs molecules into the hydrophobic core of micelles in the solution (Zhang et al., 1997). Meanwhile, in cases where soil biota was used, such as earthworms, collembola and plants, some drawbacks such as time and space consumption and high costs arise as issues (Bispo et al., 1999). In recent years, recombinant bioluminescent bacterial strains that use specific promoters fused to the bioluminescence genes (lux genes) have been applied in environmental monitoring (Heitzer et al., 1994; Willardson et al., 1998; Gu et al., 1999; Min et al., 1999). Advantages of using recombinant bioluminescent bacteria as biosensing cells include rapid responses, low costs, and improved reproducibility.

Therefore, in this study, two different techniques, immobilization of the recombinant bioluminescent bacterial cells and use of a non-toxic biosurfactant, were combined to develop an in-situ toxicity biosensor system for soil. A recombinant Escherichia coli, GC2, containing a lac::luxCDABE fusion (Marincs et al., 1994) immobilized in solid agar media was used to detect the toxicity of soil contaminants. This bacterium constitutively emits light under normal conditions. When growth and metabolism of these bioluminescent bacteria is inhibited by their exposure to toxic chemicals, the result is a loss in bioluminescence (BL). Phenanthrene was chosen as a model toxicant because it has a relatively low vapor pressure (0.018 pa at 25 °C) and water solubility (1.26 μg/ml) (Zhang et al., 1997). A biosurfactant, rhamnolipid, was used to extract phenanthrene from the soil. This biosensor kit containing immobilized cells and glass beads was connected to a fiber optic probe and inserted into a mini-bioreactor, which was maintained at a constant temperature and was aerated. The system’s optimized operating conditions and the effects of the biosurfactant on phenanthrene solubilization are presented in this paper. In addition, the method of cell immobilization and the effects of glass beads and pH on the bioluminescence of the immobilized cells are also discussed.

2. Materials and methods

2.1. Bacterial strains, chemicals, and materials

The bioluminescent bacterium GC2 is a recombinant E. coli, strain RFM443, harboring the pLITE2 plasmid (Gil et al., 2000). The plasmid, pLITE2 (lac::luxCDABE), was a gift of Dr Marincs in New Zealand (Marincs et al., 1994). The biosurfactant, rhamnolipid, was purchased from the Jeneil Biosurfactant Co. (USA). This biosurfactant is a mixture of monorhamnolipid and dirhamnolipid at an approximate ratio of 1:1. The CMC of this biosurfactant is 0.037 mM (0.002 volume percentage (% v/v)). Phenanthrene was obtained from Wako Pure Chemical Industries, Ltd. (Japan). Methylene chloride, HPLC-grade acetonitrile, and pure water were purchased from the Fisher Scientific Co. Ethanol was purchased from the Merck Chemical Co. The glass beads were purchased from the Sigma Chemical Co. The glass beads are transparent and their diameter ranges from 425 to 600 μm. Polypropylene tubes were purchased from the Turner Design Co. (USA). The outer diameter of the tubes is 7.5 mm. The Chumunjin (Kwangwon Province, Korea) standard sand was purchased from the Dongyang Science Co.

2.2. Immobilization procedure

The polypropylene tubes were prepared by cutting them to a length of 10 mm and then autoclaving. The transparent glass beads were washed with water, autoclaved, and completely dried in a drying oven (70 °C, Fisher Scientific Co. USA) before use. The glass beads were then added to the polypropylene tubes with each tube holding 0.02, 0.05, or 0.15 g. GC2 cells were grown at 37 °C in a flask with shaking at 250 rpm containing Luria Bertani (LB, DIFCO Co. USA) medium supplemented with 100 μg of ampicillin per ml until an optical density of 4.4, measured at 600 nm (OD600). Fifty milliliter of this culture were centrifuged at 6000 rpm and 25 °C for 10 min, and the media was decanted. The collected cells were resuspended with 500 μl of fresh LB medium. The agar media, or immobilizing matrix, was made from micro agar (DUCHEFA Co. Netherlands) and LB medium. After mixing 15 g/l micro agar and 25 g/l LB medium, this mixture was autoclaved and maintained at 40 °C in a water bath to be kept in a molten state during the immobilization procedure. The resuspended cells were mixed with 20 ml of the agar media and kept in the water bath. The prepared polypropylene tubes, containing glass beads, were promptly filled with 100 μl of the cell mixture. The cell mixture in the tubes was allowed to solidify at room temperature. The tube containing the immobilized cells, i.e. the biosensor kit (Fig. 1), was sealed in a
1.7 ml microtube (AxyGen, Inc. USA) and stored at 4 °C.

2.3. Enhancement of phenanthrene solubility using a biosurfactant

Hundred microgram of phenanthrene, in excess of the apparent solubility, were dissolved in 25 ml of ethanol. Five milliliter of this ethanol mixture were added to 100 ml flasks, and the ethanol was completely evaporated off at room temperature, 25 °C. Twenty milliliter of solutions containing various concentrations (0.29, 0.63, 1.23, and 1.85% v/v) of the biosurfactant were added to each of the flasks. As a negative control, water, without rhamnolipid, was added to a spiked flask to determine the solubility of the phenanthrene. The flasks were then sealed with rubber caps and the phenanthrene was extracted at room temperature while being mixed on a magnetic stirrer (800 rpm). At given time intervals, 1 ml samples were taken and centrifuged for 5 min in 1.7 ml microcentrifuge tubes at 6000 rpm to remove undissolved phenanthrene particles and the supernatants were then analyzed by HPLC.

2.4. Solubilization of soil-bound phenanthrene using the biosurfactant

Sand, as a sample soil, was sieved to a particle size ranging from 425 to 500 μm with a sieve shaker (JISICO Co., Korea). The sieved sands were washed with water, sterilized by autoclaving, and completely dried in a drying oven (Fisher Scientific Co. USA) at 70 °C. In a glass vial, 5 g of the sand were mixed with phenanthrene dissolved in methylene chloride to give a final concentration of 200 mg/kg. In order to ensure a uniform distribution of phenanthrene, the vial was placed on a rotary shaker for 30 min followed by evaporation under a hood for 1 week to remove the methylene chloride. The contaminated sands were placed in stainless steel reactors and extracted with 20 ml of rhamnolipid solutions of various concentrations (0, 0.29, 1.23 and 1.85% v/v). At given time intervals, 1 ml samples were taken from each reactor and centrifuged at 10000 rpm to remove solid particles. The supernatants were analyzed by HPLC to determine the phenanthrene concentration.

2.5. Measurement of pH

The pH value affects the activity of enzymes and the microbial growth rate. Therefore, the response of the immobilized cells may be affected by the pH value of an injected solution. The pH range suitable to the E. coli strain is from pH 6.5 to 7.0. Table 1 shows the pH values of the test sample (rhamnolipid solutions, extracted phenanthrene solutions) before injection. All of them were within the optimum range of pH.

2.6. Measurement system

The test reactor used is a 50 ml stainless steel cylinder having a water jacket to maintain a constant temperature using a heated circulation water bath (JEIO TECH, Korea). This reactor was filled with 25 ml of fresh LB medium. Filtered air was supplied through a head port with a sparging tube while excess gas was vented through an outlet port. An injection port for the test samples was also included on the head port. The biosensor kit was connected to the end of a fiber optic probe by a black rubber tube and inserted through a hole in the reactor until submerged below the LB (Fig. 2). The other side of fiber optic probe was connected to a highly sensitive luminometer (Model TD-20e, Turner Design, CA) to measure the BL from immobilized cells in the biosensor kit. The luminometer was linked to a personal computer through a RS232 serial connection in order to acquire real time data (Fig. 2). Various

Table 1
pH values of rhamnolipid solutions and extracted phenanthrene solutions

<table>
<thead>
<tr>
<th>Rhamnolipid solutions</th>
<th>pH</th>
<th>Phenanthrene concentration (ppm)</th>
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<tr>
<td>Rhamnolipid concentration (% v/v)</td>
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<tr>
<td>0.29</td>
<td>7.01</td>
<td>2.06</td>
<td>6.92</td>
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<tr>
<td>0.63</td>
<td>6.92</td>
<td>10.3</td>
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<td>1.23</td>
<td>6.90</td>
<td>17.6</td>
<td>6.93</td>
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<tr>
<td>1.85</td>
<td>6.83</td>
<td>22.5</td>
<td>6.90</td>
</tr>
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concentrations of the test samples (rhamnolipid solutions, the extracted solutions from the flasks and contaminated soils) were prepared and stored in glass vials. After the BL of the unit steadied, using a syringe, 5 ml of the test samples were injected into the reactor.

2.7. Chemical analysis

Phenanthrene concentrations in the extracted solutions were measured by high-performance liquid chromatography (HPLC). The HPLC system consisted of a HPLC pump (Waters™ 600 controller), an autosampler (Waters™ 717 plus autosampler), an UV absorbency detector (M720, Youngin Co., Korea), a differential refractometer (Waters™ 410) and a reversed-phase C18 Nova Pak column (Model WAT 086344, Waters, USA). The mobile phase consisted of a 65:35 (v/v) mixture of acetonitrile and water and had a flow rate of 1 ml/min. UV absorbance was measured at a wavelength of 254 nm. The injection volume used was 10 μl with a running time of 10 min.

3. Results and discussion

The recombinant E. coli, GC2, harboring the pLITE2 plasmid in RFM443 shows a reduction in its bioluminescent level with inhibition of the cellular metabolism brought on by toxic chemicals. The maximum response of the immobilized cells was obtained when the concentration of the cells was between $1 \times 10^9$ and $2 \times 10^9$ cells/tube (Gil et al., 2000). Thus, in this study, a concentration of $1 \times 10^9$ cells/tube was used for all experiments. The phenanthrene extracted using the biosurfactant was injected into the test reactor. In another reactor, the same concentration of rhamnolipid as used for extraction was added as a negative control. The toxicity was examined using the relative bioluminescence ($RBL = \frac{test\ BL}{control\ BL}$).

3.1. Response to different biosurfactant concentrations

In this study, rhamnolipids were used to enhance the solubility of phenanthrene. If the solvent used for extraction of the toxic chemicals has a toxic effect on the bioluminescent cells, the sensitivity of the biosensor may drop considerably. Additionally, in the case of a volatile solvent, it is difficult to perform an extraction step because there is a possibility that the solvent and the extracted chemical will be lost. It has been shown that the BL of the immobilized cells slightly decreased, less than 4%, after the injection with the same concentrations of biosurfactant as used for extraction of phenanthrene (Data not shown). Therefore, the biosurfactant should have little affect on the sensitivity of this biosensor. In addition, since the rhamnolipids is non-volatile, there is no loss of phenanthrene during the extraction.

3.2. Phenanthrene solubility

The aqueous solubility of phenanthrene in the flasks increased proportionately with an increase in rhamnolipid concentration (Fig. 3). The concentration of phenanthrene in the aqueous phase was found to rapidly increase and as higher biosurfactant doses were added for extraction, the rates of solubilization also increased. After an extraction time of about 30 min, the solubility of phenanthrene reached a saturated level and no more phenanthrene was transferred into the hydrophobic micelle core of the rhamnolipids (Fig. 3).

In the case of the extraction from the artificially contaminated soils, the aqueous solubility of phenanthrene also increased with increasing rhamnolipid conc-
centrations (Fig. 4). If no loss of the phenanthrene in the soils was assumed, about 85% of phenanthrene was extracted from the contaminated soils at the highest concentration (1.85% v/v) of rhamnolipids. However, in comparison with the solubility of phenanthrene in the flasks, the solubility of phenanthrene from the soil was lower. This could be explained by the fact that the micellization of the biosurfactant in the presence of the soil is changed due to adsorption of the biosurfactant to the soil particles, resulting in an aqueous phase concentration of biosurfactant being considerably less than that added (Laha and Luthy, 1991). However, it is apparent from the solubilization experiments that the biosurfactant significantly enhanced the phenanthrene solubility as compared to the samples in its absence.

3.3. Stability of immobilized cell bioluminescence

The immobilized cell biosensor kits were stored at 4 °C without changes in activity until used, as previously shown (Gil et al., 2000). These biosensor kits were attached to the fiber optic probe and immersed in the LB media in the test reactor, which was maintained at 37 °C. Since the immobilized cells in the biosensor kits were immersed, they would typically face some retardation of their activity, due to mainly oxygen limitation. The lack of a sufficient oxygen supply would affect the BL of the immobilized cells since it is needed for the production of light. Fig. 5 shows the effect of the aeration rate on the BL of the immobilized cells. The biosensor kits containing 0.05 g of glass beads were set in the test reactor, which was supplied with no aeration, 15 and 30 ml air/min. The BL value for the samples with no aeration and 15 ml air/min both continuously decreased. In contrast, the sample with an air flow rate of 30 ml/min achieved a steady BL value after about 40 min. This level was maintained for more than 100 min. Therefore, this flow rate was used for all subsequent experiments.

Fig. 6 shows the effect the amount of glass beads in the biosensor kits had on the stability of the immobilized cells. Separate kits containing different amounts of glass beads (no beads, 0.02, 0.05, 0.15 g) were set in the test reactor. The BL values for the kits having no glass beads and 0.02 g glass beads both decreased continuously. However, with a greater amount of glass beads (0.05, 0.15 g), the BL reached a steady-state which lasted for at least 2 h. As shown in Fig. 6, the steady state BL values were reached more rapidly with an increase in the amount of glass beads and were maintained longer. The steady-state level with 0.05 g of glass beads was obtained after 40 min and maintained its level for more than 100 min. In the case where the 0.15 g of glass beads were used, the BL value reached a steady state level within 10 min and was maintained for...
more than 150 min. This duration of the steady state levels is sufficient to detect the toxicity of phenanthrene. The shorter adaptation time and longer duration of the steady state BL seen with the increase in the amount of glass beads used may be related to the transfer of dissolved oxygen from the aqueous phase to the immobilized cells. The decrease of the BL value without glass beads may be due to a diffusion limitation of oxygen. The small, transparent glass beads enhance the porosity of immobilized matrix, here agar. Therefore, the dissolved oxygen is diffused more easily and rapidly into the biosensor kit. Since the biosensor kit with more glass beads has a greater porosity, the diffusion of oxygen is enhanced in that biosensor kit.

3.4. Effect of temperature

The bioluminescent bacteria and their enzyme activity are affected by variations in temperature. Beyond the appropriate temperature range, the BL value reduces or may not be emitted, and with that the sensitivity of biosensor may also decrease. To investigate the effect of the temperature on the BL of the immobilized cells, the BL values were measured at the various temperatures (20, 25, 30, 37, and 40 °C) in this system. After a steady BL level was reached, a 10.3 ppm phenanthrene solution was injected into the test reactor. The percent reduction in BL after 20 min is shown in Fig. 7. The percent reduction ((1 − RBL) × 100) represents the sensitivity of the biosensor (Gil et al., 2000) with the maximum percent reduction, 40.5%, in this experiment being obtained at 37 °C. The fact that the strains harboring the plasmid pLITE2 (lac::luxCDABE) were found to be most active at 37 °C has been reported previously by Marincs et al. (1994) and Gil et al. (2000). The sensitivity of the biosensor was diminished at lower temperatures. At 20 °C, a response of the biosensor to phenanthrene was not seen while at 25 and 30 °C the percent reductions observed were 7.3 and 29.8%, respectively. The percent reduction was also lower at temperatures above 37 °C. Therefore, since 37 °C gave the greatest reduction, all the following experiments were conducted at this temperature.

3.5. Response to different phenanthrene concentrations

After the BL level reached a steady state, the extracted phenanthrene solutions were injected into the test reactor. The BL values decreased rapidly at first dependant on the phenanthrene concentration and then, from about 15 min after induction, decreased

![Fig. 5. Effect of aeration on the stability of the immobilized cells bioluminescence. Immobilized cell concentration = 1 × 10^9 cells/tube. Amount of glass beads = 0.05 g/tube. Temperature = 37 °C.](image-url)

![Fig. 6. Effect of amount of transparent glass beads on the stability of the immobilized cells bioluminescence. Immobilized cell concentration = 1 × 10^9 cells/tube. Aeration rate = 30 ml air/min. Temperature = 37 °C.](image-url)

![Fig. 7. Effect of temperature on the relative response ratio. Immobilized cell concentration = 1 × 10^9 cells/tube. Amount of glass beads = 0.05 g/tube. Aeration rate = 30 ml air/min. Temperature = 20, 25, 30, 37 and 40 °C.](image-url)
Fig. 8. Response to different phenanthrene concentrations. Biosurfactant concentration used for phenanthrene extraction = 1.85% v/v. Immobilized cell concentration = $1 \times 10^9$ cells/tube. Amount of glass beads = 0.05 g/tube. Aeration rate = 30 ml air/min. Temperature = 37 °C.

constantly at almost the same rate (Fig. 8). As the concentration of the injected phenanthrene was increased, the initial rate of BL loss also increased. As the micelles with phenanthrene in their hydrophobic core are transported to the cells, the hydrophobic part of micelle forms a hemi-micelle and attaches to the cell and the chemical in the micelle diffuses into the cell (Guha and Jaffe, 1996a). The phenanthrene in the micelle of the rhamnolipids would then be transferred into the immobilized cells. As soon as the phenanthrene solution was injected, the immobilized cells near the opening of the tube were exposed to this toxic chemical and their BL values abruptly decreased. In the biosensor kit, the micelles with phenanthrene might be transported slowly through pores of the solid agar matrix. Thus, the BL of some immobilized cells continued to slowly decrease after the initial drop in the BL. Fig. 9 shows the dose dependent response for phenanthrene 15 min post-injection.

To investigate the response of the extracted solution from artificially contaminated soils, the sorbed phenanthrene was extracted with 1.85% v/v of rhamnolipids for 30 min and 5 ml of the extracted solution was injected into the test reactor. This solution was also analyzed by HPLC. The concentration of phenanthrene in the test reactor was determined to be 4.85 ppm. The RBL value for this solution was measured as $0.82 \pm 0.04$ at 15 min after injection. Using this value and Fig. 9, the phenanthrene concentration in the test reactor was calculated as $5.52 \pm 1.27$ ppm.

4. Discussion

The detection of toxicity with this biosensor might be affected by several factors, such as the transport of hydrophobic phenanthrene from soil to the cells, the diffusion of dissolved oxygen, the temperature and the immobilized cell mass. The optimum immobilized cell mass was between $1 \times 10^9$ and $2 \times 10^9$ cells/tube (Gil et al., 2000). Since this biosensor kit is being immersed during measurement, the immobilized cells might detach and be released from the immobilizing matrix under the mixing condition. We have tested the detachment of the cells from the immobilizing matrix during operation of the biosensor kit, and found that only about 0.5% of the total number of the immobilized cells were released during 100 min (data not shown). Thus this amount of the cells released during such a long time should be negligible considering the fact that measurement of toxicity is usually completed in 30 min.

This biosensor showed good responses in the temperature range of 30–37 °C. Therefore, the detection of soil contaminants with this biosensor would be best if the temperature is maintained within this range. When the phenanthrene, extracted with the biosurfactant solution, was injected into the test reactor, the BL of the immobilized cells decreased rapidly due to the contacts between the micelles with phenanthrene and the immobilized cells located at the opening of the biosensor kit. As well, the micelles with phenanthrene might be sorbed onto the pores of immobilizing matrix. Therefore, the transfer rate of phenanthrene to the cells was reduced and, consequently, the BL value decreased with a slower rate than that of the initial decrease.

The diffusion limitation of oxygen also deteriorates the stability of the immobilized cell bioluminescence and the activity of their enzymes. The addition of small, transparent glass beads advances the diffusion capacity of the dissolved oxygen. In addition, the mi-
cielle with phenanthrene may be more easily transferred into the biosensor kit due to an increase in the porosity of the immobilized matrix, and, thus, the sensitivity of the system is enhanced. It is difficult to detect hydrophobic toxic chemicals sorbed in soils using a whole cell biosensor due to their insoluble property. In fact, we have tested the possibility of using this biosensor kit without using a biosurfactant and found that a decrease in the BL did not occur (data not shown). However, the use of a biosurfactant can enhance the transfer and bioavailability of hydrophobic chemicals. Thus, this biosensor system would be useful in the detection of soil contaminant toxicity. The minimum detection level of this biosensor was 2 ppm of phenanthrene in the aqueous phase. Setting the most optimum sensing conditions, such as optimum size and amount of glass beads, would lower this level. To determine more critically the degree of soil contamination from the responses of immobilized cells, a more active, accurate correlation between the extraction from contaminated soils and the response of this biosensor is needed.

In this study, a constitutive bioluminescent bacterium was used for the detection of toxicity. The BL value of these bacterial strains decreases whenever the cells are exposed to toxic conditions, regardless of the mode of toxicity. Therefore, if the strains with stress or chemical specific promoters, induced by particular toxic chemicals, are used as an immobilized cell, specific toxic chemicals in contaminated soils can be detected and quantified.

5. Conclusion

A biosensor for the detection of hydrophobic soil contaminants was developed. A dose dependent response for phenanthrene as a sample toxic chemical was shown. The glass beads added to the immobilizing matrix were found to prolong the maintenance of the steady state BL. Using a biosurfactant, here rhamnolipid, enhanced the mass transfer rate of phenanthrene from the contaminated soil. This biosensor was able to detect the toxicity of phenanthrene rapidly and reproducibly, and, therefore, can be applied to a field investigation as an in situ biosensor due to its small size and easy operation.

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