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Application of stimulating agents on the immobilized bioluminescence strain *Pseudomonas putida* mt-2 KG1206, preserved by deep-freezing, for the convenient biomonitoring

In Chul Kong

Department of Environmental Engineering, Yeungnam University, Gyongsan Kyungbuk 712-749, Korea.
E-mail: ickong@ynu.ac.kr

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Abstract

This study was conducted to develop methods for the application of an immobilized bioluminescence strain (KG1206), preserved by deep-freezing (DF), for the monitoring of contaminated environments. The immobilized cells, preserved by DF, required approximately 2 hr for reconstitution of their activity. A large reduction in bioluminescence was observed due to the DF process; 0.07–0.58 times that of the non deep-frozen (NDF) immobilized strain. The decreased bioluminescence activity induced by the DF process was enhanced by the stimulants, sodium lactate (SL) and KNO_3. However, regardless of the inducer chemical tested, the immobilized strain modified with KNO_3 consistently produced greater bioluminescence than that treated with SL, in the range of 3.0–10.7 (avg. 6.7 ± 3.69) and 1.2–4.2 (avg. 2.4 ± 1.47) times that of control, respectively. All KNO_3 treatments of contaminated groundwater samples also resulted in an increase in bioluminescence activity, but the rate of stimulation varied for each sample. Also, no strong linear correlation was observed between the bioluminescence and the total concentration of an inducer, which may related to the complex characteristics of the environmental samples. Overall, the results demonstrated the ability of immobilized genetically engineered bacteria, preserved by DF, to measure a specific group of environmental contaminants using a stimulating agent (KNO_3), suggesting the potential for its preliminary application in a field-ready bioassay.

Key words: bioluminescence; biomonitoring; deep-freezing; groundwater; immobilization

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Introduction

Petroleum hydrocarbons (PHCs) are widely used and commonly found in environments, such as groundwater underlying polluted sites (Breweley et al., 1990; West et al., 1995). The United States Environmental Protection Agency (US EPA), the Quebec Ministry of Environment (MENV) and Organizations of Environment and Public Health around the world have treated PHCs as significant contaminants (Zhou and Crawford, 1995). Thus, the development of methods that can be used for the rapid monitoring or assessment of contaminated sites has gained interest. Several microbial processes can be used in biomonitoring assays, and such microbially-derived assessment tools are usually simple, rapid and cost effective compared to conventional chemical techniques.

Under appropriate conditions, the recombinant bioreporter gene can produce a direct signal correlated to the concentration of a specific pollutant (Burlage, 2002). Such biosensors can generate valuable information regarding the bioavailability of contaminants in the environment, as well as serve as useful alternatives to more expensive chemical methods for *in situ* monitoring or on-site analysis. For environmental application, it is important that the strains of bacteria exhibit high bioluminescence intensity and are portable. For example, the genetically engineered bioluminescent bacterium, *Pseudomonas putida* mt-2 KG1206, has shown a great feasibility for use as a tool in biomonitoring of PHCs in the environment (Ko and Kong, 2009). This strain contains a plasmid, where, P_m, the lower promoter of the TOL-plasmid *xyl*-gene, is recombined into the upper part of the *lux*-gene of vector plasmid, pUCD615 (17.7 kb). When a regulatory factor, produced by inducing chemicals, controls the expression of P_m, bioluminescence is released by the *lux*-gene. Therefore, in the presence of toluene analogs, KG1206 show bioluminescence activity during the degradation of compounds (Kong, 2006).

For the application of such strains, the cells can be immobilized and deep-frozen (DF) if necessary. Cells could be immobilized in natural and synthetic polymers. The immobilization of microorganisms in a polymeric matrix has several benefits, including regeneration and reuse of biomass and easy carrying and storage for environmental use (Vijayaraghavan et al., 2007). There are many reports on the use of immobilization methods for microorganisms and enzymes in biological technologies. Immobilization

can be performed by four different methods: carrier-binding, cross-linking, entrapping and a combination of all three. Alginate, carrageenan, polyacrylamide and glass beads are commonly used as a carrier for the cells (Stormo and Crawford, 1992). This immobilized strain could be preserved by DF for its more practical use in the field, allowing maintenance and easy distribution (Choi and Gu, 2003). However, the effects on the cell activity by such processes need to be investigated.

In this study, the activity of the immobilized bioluminescence strain, KG1206, preserved by DF, was investigated with pure inducer pollutant and contaminated groundwater samples. In addition, a better stimulant, which enhances the bioluminescence activity, was continuously investigated in the presence of pure inducer chemicals. The determined procedure was applied to environmental groundwater samples contaminated with PHCs.

1 Materials and methods

1.1 Culture conditions and bioluminescence measurement

P. putida mt-2 KG1206 produces bioluminescence in the presence of appropriate inducing compounds, and is also capable of degrading analogs and catabolic intermediates of toluene, including *m*-toluate and benzoate (Kong, 2006). The KG1206 strain was maintained and stored using standard procedures (Sambrook et al., 1989). Cultures were stored at –70°C until needed, at which time they were grown overnight in Luria-Bertani (LB) medium, supplemented with 50 mg/L of kanamycin, at 27°C with shaking (130 r/min). A 1:30 dilution was then made up in LB medium, with the culture grown to an optical density (OD_{600}) of approximately 0.6. The LB medium consisted of 10 g tryptone, 5 g yeast extract, 5 g NaCl and 0.5 mL of 2 mol/L NaOH per liter of broth, with 50 mg/L kanamycin to prevent bacterial contamination. The inducers including *m*-toluate, toluene and xylene isomers were tested. All reagents, antibiotics and test chemicals were obtained from Sigma Chemical (USA) or Aldrich Chemicals (USA). The bioluminescence intensity was measured using a Turner 20/20 Luminometer (Turner, USA), where the maximum limit of detection was 9999 relative light units (RLU).

1.2 Immobilization and DF of KG1206 strain

Subcultured KG1206 strain was immobilized using alginate polymer ($(C_6H_8O_6)_n$; DC Chemical Co., Ltd., USA). Equal mixtures of KG1206 culture (OD_{600} = 0.6) and 24% sucrose were completely mixed, with an equal volume of 2% alginate polymer, in test vials and then transferred to a syringe, without a needle. This mixture was injected into 0.1 mol/L cold $CaCl_2$ solution and left in a refrigerator for 2 hr to allow strong bead formation. Approximately 25 beads were formed from 1 mL of the mixed solution, corresponding to 0.04 mL mixture per bead. After decanting the $CaCl_2$ solution, the alginate beads were stored at –70°C until needed. The DF immobilized strain was reconstituted before use.

1.3 Application of immobilized strain, preserved by DF

Immobilized beads, preserved by DF, were reconstituted prior to use. The appropriate time to recover cell activity (reconstitution time) was determined after 0.5, 1, 2 and 3 hr in a water bath. The bioluminescence activity under each condition was compared in the presence of various inducers, with control sets (NDF).

The appropriate enhancer of the bioluminescence of the reconstituted immobilized strain was determined. The effects of sodium lactate (24 μL: 2 g carbon/L) and potassium nitrate (0.04 g: 20 g chemical/L) were assessed by their addition into a vial containing reconstituted immobilized beads, with a direct inducer; *m*-toluate (to a final concentration of 1 mmol/L), in a vial. As a control, immobilized cells without any enhancer were prepared. The agent determined to have the greatest stimulatory effect on the bioluminescence activity of the immobilized cells was then tested using other inducers (toluene and xylene isomers), as well as environmental groundwater samples.

Groundwater samples containing petroleum hydrocarbons were collected from contaminated sites in Korea (Ko and Kong, 2009). The bioluminescence activities were measured after transferring 100 reconstituted beads into serum vials supplemented with groundwater samples. The bioluminescence activity was compared both with and without a stimulant in the samples. The serum vials were sealed with Teflon septa to avoid the loss of volatile compounds, and then incubated at 27°C with shaking (130 r/min) for 3–4 hr. Four beads and 400 μL solution were transferred into sample tubes for detection. All tests were performed in triplicates.

2 Results and discussion

2.1 Bioluminescence activity of the immobilized strain, preserved by DF

KG1206 contains the intact TOL plasmid and a P_m-*lux* fusion plasmid. P_m is the promoter of the lower or *meta* operon (13 genes) of the TOL catabolic plasmid, pWW0, is responsible for producing bioluminescence in the presence of toluene analogs and their metabolites (Harayama and Rekik, 1990). Before using the immobilized strain stored in a deep freezer, the influence of the reconstitution time on the recovery of the bioluminescence activity was examined after 0.5, 1, 2, and 3 hr of reconstitution. Following reconstitution, the bioluminescence activity over a period of 5 hr was examined using *m*-toluate as an inducer (Fig. 1). The reconstitution time appeared to affect the maximum bioluminescence activity, as well as the bioluminescence intensity. For example, the maximum bioluminescence appeared after 5 hr (2108 ± 736.8 RLU), 4 hr (3420 ± 585.5 RLU), 3 hr (2918 ± 287.8 RLU), and 2 hr (2220 ± 109.6 RLU) of incubation for the sets with reconstitution time 0.5, 1, 2, and 3 hr, respectively. With the exception of the shortest reconstitution time, 0.5 hr, sets reconstituted for a short time produced high bioluminescence intensity. The times required for both high activity and biolumines-

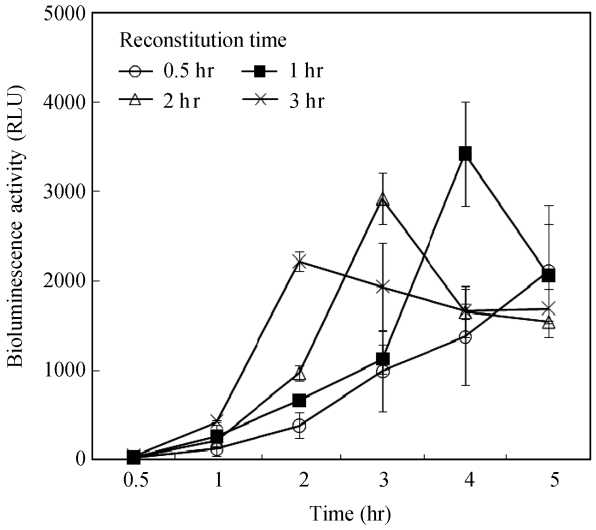

Fig. 1 Time course of the bioluminescence activity of immobilized KG1206, preserved by DF, following different reconstitution time (inducer 5 mmol/L *m*-toluate).

cence intensity are important factors in the development of a bioassay method. Therefore, the appropriate reconstitution time should be determined, with respect to the time required for a bioassay and the development of the bioluminescence intensity. In this study, a reconstitution time of 2 hr was determined as being reasonable for further investigation. However, this condition can be adjusted depending on the cell condition and experimental situation, etc.

Following 2 hr of reconstitution, the bioluminescence activities of the immobilized beads were compared with various inducer pollutants (1 mmol/L of *m*-toluate, toluene, and xylene isomers). Figure 2 shows the total (6 times measurement: 0.5, 1, 2, 3, 4, 5 hr) and maximum bioluminescence produced during 5 hr of incubation. Of the tested chemicals, *m*-toluate is known to directly activate XylS protein, while the other compounds are activators of XylR, and converted to lower pathway inducers during catabolism. As expected, weak expressions were observed compared to those found with the subcultured cells, but fully detectable bioluminescence did appear. The magnitude of total and maximum bioluminescence of the tested chemicals appeared in the following order: *m*-toluate > *m*-xylene ≈ *p*-xylene > toluene > o-xylene. Different patterns were observed with the subcultured (not immobilized) strain. Generally, toluene and *p*-xylene were strong and weak inducers with the subcultured strain, respectively, but contrary results were observed, with weak and strong expressions observed for toluene and *p*-xylene, respectively. The cause of this contrary result is not clear at this point, and this result will be investigated more specifically on a molecular basis.

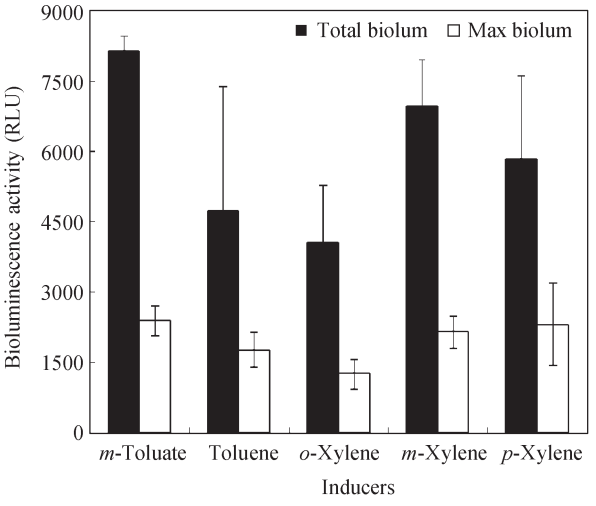

Fig. 2 Total (5 hr incubation) and maximum bioluminescence produced by the immobilized KG1206, preserved by DF, with different inducers (1 mmol/L) following 2 hr reconstitution. The range of the error bars: ± standard deviation of the bioluminescence (triplicate observation).

2.2 Effects of DF on bioluminescence activities of the immobilized strain

One primary interest was to observe the effects of the DF storage process on the bioluminescence activities of the immobilized strain. The effects by the DF process were initially examined with a strong direct inducer, *m*-toluate (5 mmol/L) (Fig. 3). The highest bioluminescence activity was induced after 3 hr of incubation, and lasted longer than approximately 5 hr for the NDF strain. The reconstituted strain clearly produced less bioluminescence, possibly due to the DF process. A slow response also occurred during the early incubation period. The bioluminescence activities after 3 hr of incubation were (9710 ± 460.2) RLU for NDF and (3567 ± 436.8) RLU for DF strain. These results suggest a reduction in the bioluminescence activity due to the DF process.

The effects of the DF process on the bioluminescence activity were further tested with various direct and indirect inducers (1 mmol/L of *m*-toluate, toluene, and xylene isomers). Figure 4 shows the total bioluminescence produced during 5 hr incubation periods with DF and NDF immobilized beads. Of the tested chemicals, *m*-toluate is known to directly activate XylS protein, while the other compounds are activators of XylR, and converted to lower pathway inducers during catabolism. Weak bioluminescence expressions of the reconstituted DF cells were observed

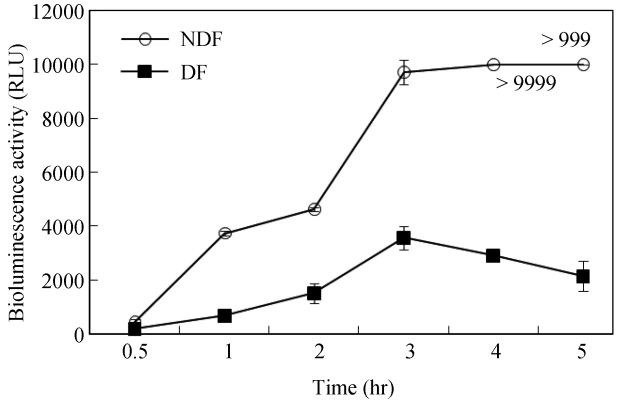

Fig. 3 Effects of the DF preservation process on the bioluminescence activity of immobilized KG1206 with 5 mmol/L of *m*-toluate. NDF: non deep-frozen; DF: deep-frozen.

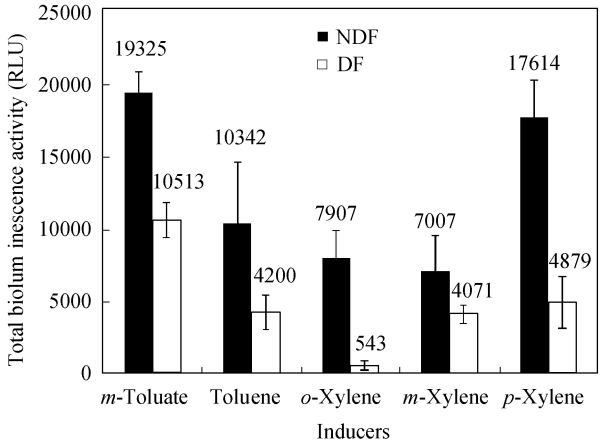

Fig. 4 Comparison of the bioluminescence activities between the NDF and DF immobilized strain in the presence of various inducer chemicals (1 mmol/L). Values are the sum of the bioluminescence produced, measured after 0.5, 1, 2, 3, 4 and 5 hr incubation periods.

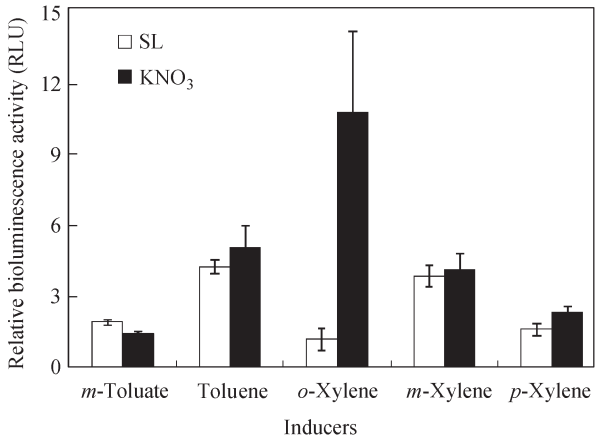

Fig. 5 Effects of SL and KNO_3 on the bioluminescence activity of KG1206 with individual inducers. Values are the mean of relative bioluminescence, measured at incubation time 0.5, 1, 2, 3, 4 and 5 hr. Error bars: standard deviation of the bioluminescence (triplicate observation).

compared with those of the NDF cells, but fully detectable bioluminescence did appear. The bioluminescence activities of the DF cells were within the range 0.07–0.58 times those of the NDF cells. For example, the total bioluminescence of the NDF and DF immobilized cells were 19,325 and 10,513 RLU with 1 mmol/L *m*-toluate, respectively. The magnitude of the total bioluminescence of the tested chemicals during 5 hr incubation periods appeared in the following order: *m*-toluate > *p*-xylene > toluene > *m*-xylene > *o*-xylene, which was similar to those subcultured, with the exception of the activity of *p*-xylene. Both conditions showed similar patterns. In general, weak induction was observed with xylene isomers in the subcultured strain, but strong induction appeared with *p*-xylene after immobilization, regardless of the use of DF and NDF cells. The cause for this is unclear at this point, and further specific investigations at the molecular level will be required. It is possible that this decreased activity was due to cell damage during the DF and reconstitution processes (Choi and Gu, 2003). This result indicates the importance of a stimulant on the bioluminescence activity of the DF strain for environmental use.

2.3 Effects of stimulants on the bioluminescence activity of immobilized bacteria, preserved by DF

The stimulation of the low bioluminescence activity of the reconstituted DF immobilized strain is necessary for the indicator bacteria to be useful for environmental applications. The influence of the addition of SL or KNO_3 on the DF immobilized strain was investigated in the presence of 1 mmol/L of toluene, *m*-toluate, or the xylene isomers. These two chemicals are known as good stimulants of the bioluminescence activity of the KG1206 strain (Ko and Kong, 2009). Figure 5 shows the relative bioluminescence activity during a 5 hr exposure period in the presence of stimulant chemicals with inducers. Stimulation of the bioluminescence activity of P_m-*lux* gene was observed with both chemicals, but a better stimulation was mostly observed with the addition of KNO_3. Similar results were also observed with the subcultured cells, as well as with the NDF immobilized cells (data not shown). Stimulations by SL and KNO_3 were within the range of 1.2–4.2 (avg. 2.5 ± 1.38) and 1.4–10.7 (avg. 4.6 ± 3.63) times those of the control, respectively, based on the average bioluminescence produced. Relative stimulation intensity varied depending on the inducer chemicals. The highest relative stimulation was observed with *o*-xylene in the presence of KNO_3 stimulant (10.7 ± 3.40; avg. 964 RLU), but the highest bioluminescence activity was with *m*-toluate inducer, showing 3221; 6066; and 4512 RLU with control, SL, and KNO_3, respectively.

The stimulating chemicals may have caused positive effects on the bioluminescence regulatory systems, such as the *rpo*-S gene or other unidentified regulatory systems (Miura et al., 1998). Holtel et al. (1994) also reported the stimulating effects of additional carbon sources (succinate, citrate, glycerol and fructose) on the benzyl alcohol-induced activation of *xyl* promoters, while glucose, gluconate, lactate and acetate inhibited the activation of P_u. A number of mechanisms could potentially bring the stimulation and inhibition effects, ranging from changes in promoter DNA supercoiling, which may affect the gene expression in response to nutritional changes, to the specific RNA polymerase subunit, σ^{54}, for transcription (Assinder and Williams, 1990; Balke and Gralla, 1987). In author's previous investigation with various nitrogen compounds, a greater stimulation by nitrate forms, KNO_3 and NH_4NO_3 was observed, while a complete inhibition was with KNO_2. Also no significant effect was observed with other ammonium forms, such as $(NH_4)_2SO_4$ and $(NH_4)_2C_2H_4H_2O$ (data not shown). Based on the results, KNO_3 was chosen for further investigation with environmental samples.

2.4 Effects of stimulant KNO_3 on immobilized KG1206, preserved by DF, with contaminated groundwater samples

The effect of the bioluminescence stimulant (KNO_3) was observed using environmental samples contaminated with PHCs. This investigation was performed to evaluate

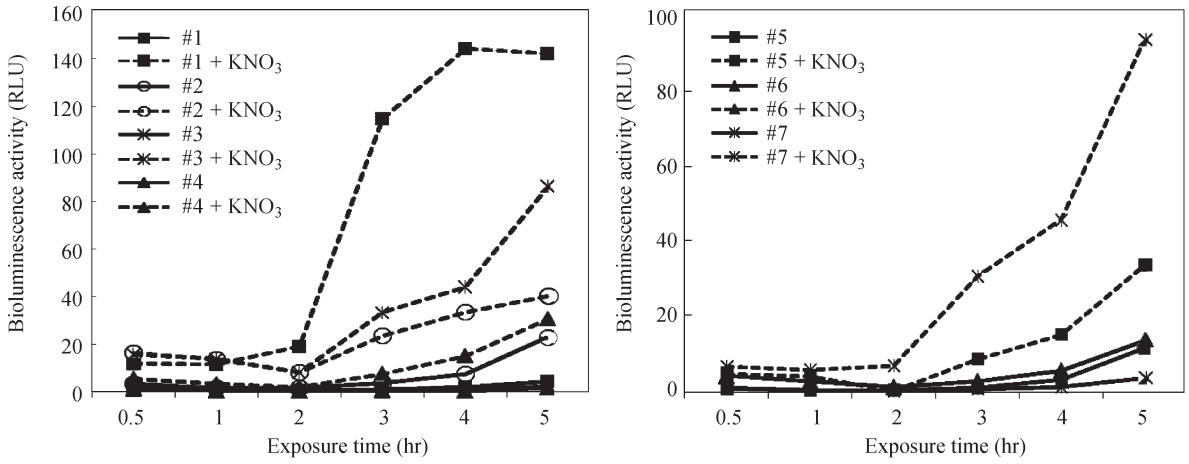

Fig. 6 Effect of KNO_3 on the bioluminescence activity of DF immobilized strain, KG1206 with contaminated groundwater samples.

the applicability of the immobilized strain, preserved by DF, combined with a bioluminescent stimulant on groundwater samples containing various pollutants, including inducers, i.e., toluene and xylene isomers. The concentrations of the major contaminants in groundwater samples are shown in Table 1. The total inducer concentrations ranged from 0.0 to 26.2 mg/L, but a wide range of compounds was contained within the samples. Of the contaminating compounds, high concentrations of the octane booster, methyl tertiary butyl ether (MTBE), were generally found, especially in samples #2 (250 mg/L) and #6 (580 mg/L). Without the addition of KNO_3, the bioluminescence activity was generally very low (maximum 23 RLU; solid line on Fig. 6). The possible reasons for this low bioluminescence as follows: the low level of inducers in the environmental samples, inhibition by other contaminating chemicals, and the limit of diffusion of these into the entrapped cells or cell damage during the reconstitution process, etc. All KNO_3 treatments resulted in an increase bioluminescence activity (maximum 290 RLU; dotted line in Fig. 6), but the rate of stimulation varied for each sample. Also, no strong linear correlation was observed between the bioluminescence and the total concentration of an inducer, which may have been related to the complex characteristics of the environmental samples. Some false positives (high bioluminescence with a low inducer concentration) and false negatives (low bioluminescence with a high inducer concentration) were observed in these experiments. These were likely to have been occurred by the inhibition or stimulation of the bioluminescence activity by the presence of unknown inducer or suppressor chemicals in the environmental samples; however, the causes are currently unclear. This correlation will be further investigated with a wide range of samples. The causes of the interference will also need to be identified, with a correction made when assessing contaminated samples. Nevertheless, the immobilized KG1206 with a stimulant agent, preserved by the DF process, presented in this research has shown potential as a portable assay for the preliminary on-site monitoring of specific inducer contaminants, especially with a stimulant agent.

Table 1 Concentrations of the inducers and important contaminants in groundwater samples

Sample	Pollutant concentration (mg/L)		
	Inducers[a]	MTBE	Benzene
#1	15.9	95	0.4
#2	20.3	250	3.6
#3	26.2	35	12
#4	0.01	1.6	DL
#5	DL	4.8	DL
#6	5.8	580	5.2
#7	DL	0.25	DL

[a] Inducers are the sum of the concentrations of toluene and the three xylene isomers. DL: below the detection limit.

3 Conclusions

To develop a portable biomonitoring assay, using an immobilized bioluminescence bacteria preserved by DF, several factors were investigated, such as an appropriate reconstitution time, chemicals suitable for the stimulation of bioluminescence, and the effects of these stimulants on strains using both the pure chemicals and groundwater samples. The results indicated that, although the DF immobilized strain produced low bioluminescence intensity, detectable bioluminescence was observed during the exposure period. Both SL and KNO_3 stimulated the bioluminescence activity of the reconstituted strain, but a greater increase in the bioluminescence was observed with KNO_3. This suggested that the low bioluminescence activity exhibited by the DF immobilized strain may be stimulated by amending the culture with an appropriate agent, such as KNO_3. The bioluminescence activity of the reconstituted cells in the presence of groundwater samples was also stimulated by the addition of KNO_3. These findings might be applicable to other recombinant bioluminescence strains for environmental monitoring. Overall, the results demonstrated the ability of immobilized engineered bacteria, preserved by DF, for the measurement of a specific group of environmental contaminants using a stimulating agent, suggesting the potential for its preliminary application in a field-ready bioassay.

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References

Assinder S J, Williams P A, 1990. The TOL Plasmid: Determinants of the catabolism of toluene and the xylenes. *Advanced Microbiol Physiology*, 31: 1–69.

Balke V L, Gralla J D, 1987. Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. *Journal of Bacteriology*, 169: 4499–4506.

Brewley R J F, Ellis B, Rees J F, 1990. Development of a microbiological treatment for restoration of oil contaminated soil. *Land Degradation Rehabilitation*, 2: 1–11.

Burlage R S, 2002. Emerging technologies: bioreporters, biosensors, and microprobes. In: Manual of Environmental Microbiology (2nd ed.). ASM, Washington DC. 147–157.

Choi S H, Gu M B, 2003. Toxicity biomonitoring of degradation byproducts using freeze-dried recombinant bioluminescence bacteria. *Analytica Chimica Acta*, 481(2): 229–238.

Harayama S, Rekik M, 1990. The meta cleavage operon of TOL degradative plasmid pWWO comprises 13 genes. *Molecular General Genetics*, 221: 113–120.

Holtel A, Marques S, Mohler I, Jakubzik U, Timmis K N, 1994. Carbon source – dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. *Journal of Bacteriology*, 176(6): 1773–1776.

Kong I C, 2006. An optimization of a bioassay for toluene analogs using bioluminescence reporter strain KG1206. *Soil and Sediment Contamination*, 15: 231–239.

Ko K S, Kong I C, 2009. Conditions required for the stimulation of bioluminescence activityof the genetically engineered bacteria, *P. putida* mt-2 KG1206, preserved by deep-freezing. *Science of the Total Environment*, 407: 2427–2430.

Miura K, Inouye S, Nakazawa A, 1998. The *rpoS* gene regulates OP2, an operon for the lower pathway of xylene catabolism on the TOL plasmid, and the stress response in *Pseudomonas putida* mt-2. *Molecular General Genetetics*, 259: 72–78.

Sambrook J, Fritsch E F, Maniatis T, 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Stormo K E, Crawford R L, 1992. Preparation of encapsulated microbial cells for environmental applications. *Applied and Environmental Microbiology*, 58: 727–730.

Vijayaraghavan K, Han M H, Choi S B, Yun Y S, 2007. Sorption of Reactive Black 5 by Corynebacterium glutamicum biomass immobilized in alginate and polysulfone matrices. *Chemosphere*, 68: 1838–1845.

West O R, Siegrist R L, Mitchell T J, Jenkins R A, 1995. Measurement error and spatial variability effects on characterization of volatile organics in the subsurface. *Environmental Science and Technology*, 29: 647–656.

Zhou E, Crawford R, 1995. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. *Biodegradation*, 6: 127–140.