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A biosensor for the detection of gas toxicity using a recombinant bioluminescent bacterium

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Abstract

A whole-cell biosensor was developed for the detection of gas toxicity using a recombinant bioluminescent *Escherichia coli* harboring a *lac::luxCDABE* fusion. Immobilization of the cells within LB agar has been done to maintain the activity of the microorganisms and to detect the toxicity of chemicals through the direct contact with gas. Benzene, known as a representative volatile organic compound, was chosen as a sample toxic gas to evaluate the performance of this biosensor based on the bioluminescent response. This biosensor showed a dose-dependent response, and was found to be reproducible. The immobilizing matrices of this biosensor were stored at 4°C and were maintained for at least a month without any noticible change in its activity. The optimal temperature for sensing was 37°C. A small size of this sensor kit has been successfully fabricated, and found to be applicable as a disposable and portable biosensor to monitor the atmospheric environment of a workplace in which high concentrations of toxic gases could be discharged.

Keywords: Gas toxicity; Whole cell biosensor; Recombinant bioluminescent *Escherichia coli*; Benzene

1. Introduction

Serious discharges or leaks of volatile chemical compounds were found to cause deleterious damage to lives and serious environmental problems. Some chemical compounds, such as volatile organic compounds, chlorinated derivatives, poly hydrocarbons and so forth, are very toxic to humans and cause abnormal health effects. Among them, toxic gaseous chemicals may cause serious human health problems and are involved in such groups as carcinogenics, genotoxics, developmentals, reproductives, systemic toxics, skin/eye irritant, etc. (Calabrese and Kenyon, 1991). These toxic gaseous chemicals could be exposed to the workers via air diffusion in chemical production facilities or in workplaces where such toxic chemicals are used (Ghim et al., 1998). Therefore, a great variety of physical and chemical gas sensors have been developed for detection of harmful gases (Petty, 1995; Brook and Narayanaswamy, 1997). However, some of these gas sensors are not sensitive and have a poor selectivity in the analysis of the gas mixture. Also, it is very difficult to follow the complicated procedures that should be taken for the measurement of toxic gases.

Recently, studies on biosensors that use live organisms or biological materials have been done for the detection of toxic chemicals. These biological sensors have been applied in environmental monitoring, and efforts in developing a biosensor for the monitoring of gaseous chemicals have been pursued. For instance, electrochemical biosensors using immobilized enzymes as a sensing element (Dennison et al., 1995; Park et al., 1995; Hammerle and Hall, 1996; Kaisheva et al., 1997) or whole cell biosensors (Lee and Karube, 1996; Naessens and Minh, 1998) have been developed for the detection of gaseous chemicals, and typically demonstrated a high sensitivity and selectivity. In addition, a rapid response allowing a reduction in the detection time was an another feature.

As a whole cell biosensor, use of genetically-engineered bioluminescent bacteria has recently flourished with applications in many different areas (Applegate et

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al., 1998; Gu et al., 1999). 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). Advantages of using bacterial bioluminescence as a reporter is that bioluminescence can be measured simply through the use of a fiber optic probe and light accessing equipment.

However, gas sensors developed were only able to detect the content of a specific gas, not its toxicity. There is, therefore, a clear need for sensitive, selective and reliable sensors for gas toxicity. Here, we used a recombinant *Escherichia coli*, GC1, harboring a plasmid-borne *lac::luxCDABE* fusion (pLITE2 — Marincs and White, 1994) with RFM443 as a host with the loss of bioluminescence expected to correspond with the toxicity. The toxicity of chemicals can be evaluated through the bioluminescent reaction as it reduces in intensity when the cells experience toxic or lethal conditions. This whole cell biosensor was fabricated, using an immobilization technique utilizing solid agar medium, for the measurement of toxicity through direct contact with the gas. The supplement of nutrients through agar medium could maintain the activity for a prolonged period and allow cells, embedded in the solid medium, to directly detect gaseous toxicity. The biosensor also could be produced in a compact size, which could be used as a portable biosensor. The biosensor, though

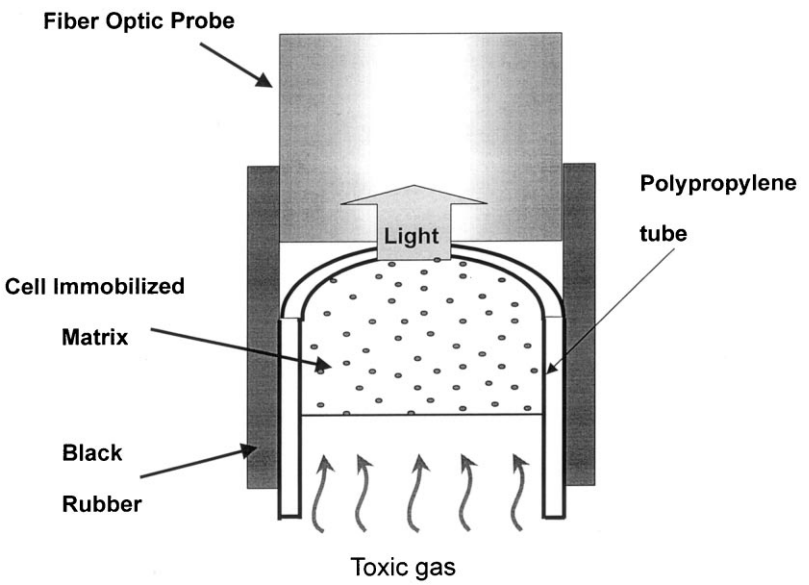

Fig. 1. Schematic diagram of the immobilized cell matrix (biosensor kit) connected with a fiber optic probe.

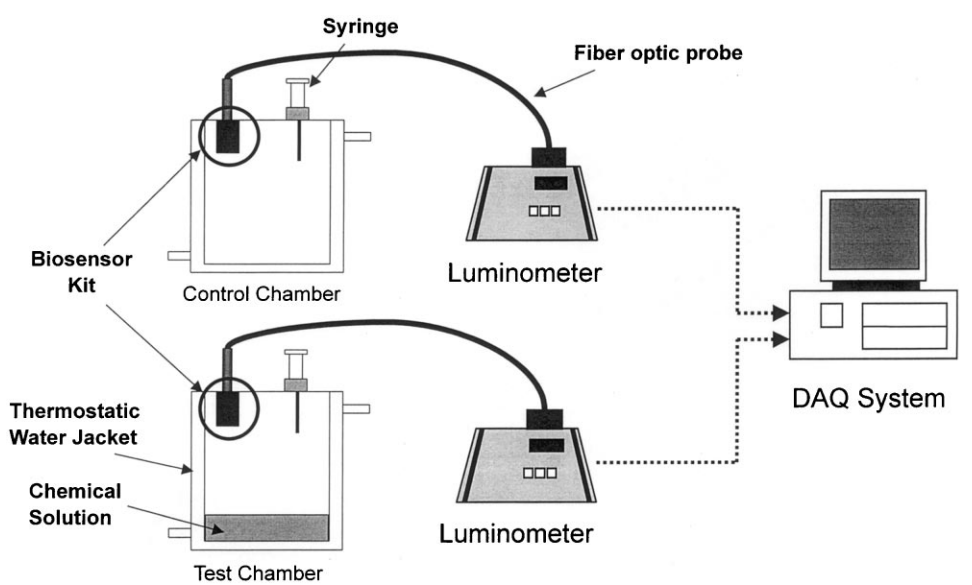

Fig. 2. BL measurement system consisting of a chamber connected with fiber optic probe and biosensor kit.

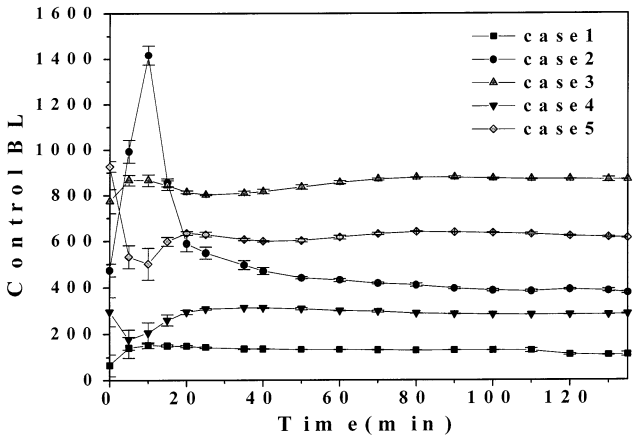

Fig. 3. The steady state BL level for different concentrations of immobilized cells; cells were stored at 4°C and tested at 37°C; case $1 = 1 \times 10^7$, case $2 = 1 \times 10^8$, case $3 = 1 \times 10^9$, case $4 = 2 \times 10^9$, and case $5 = 7.3 \times 10^9$ cells/tube.

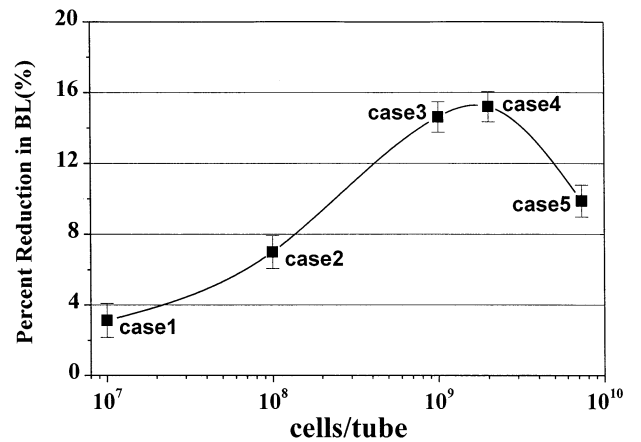

Fig. 4. Response ratios for different concentrations of immobilized cells; a 2% benzene solution was injected; the temperature was maintained at 37°C; case $1 = 1 \times 10^7$, case $2 = 1 \times 10^8$, case $3 = 1 \times 10^9$, case $4 = 2 \times 10^9$, and case $5 = 7.3 \times 10^9$ cells/tube. Percent reduction in bioluminescence = ([steady-state BL − test BL]/[steady-state BL] × 100).

small, has shown to be sensitive to gaseous vapors. The applicable features of this biosensor for toxicity monitoring of gases in the atmospheric environment were demonstrated with benzene vapor. In addition, the optimal temperature and the minimum detection level of benzene toxicity have been elucidated with this biosensor. Finally, further modification and enhancement of sensitivity are also discussed.

2. Experimental

2.1. Strains and chemicals

The plasmid used in this experiment, pLITE2 (*lac::luxCDABE*) was sent from Dr Marincs (New Zealand) and was transformed into the host strain, RFM443 in our lab to give strain GC1. As a sample toxic gas, benzene, known as a representative volatile organic compound, was chosen and purchased from the Merck Company. Oleic acid was purchased from the Kanto Chemical (Japan). Benzene solutions and its concentrations were prepared by mixing with oleic acid.

2.2. Immobilization

All strains were stored at − 70°C before being streaked on agar plates to obtain isolated colonies. One colony was inoculated into a flask containing 100 ml of Luria Bertani (LB, initial pH of 7) broth supplemented with 100 μg of ampicillin per ml. The bacteria were grown to the late exponential phase at 37°C with shaking at 300 r.p.m. The cells were collected by centrifugation at 6000 r.p.m. for 10 min at 25°C and resuspended with 100 μl of fresh LB medium. Solid

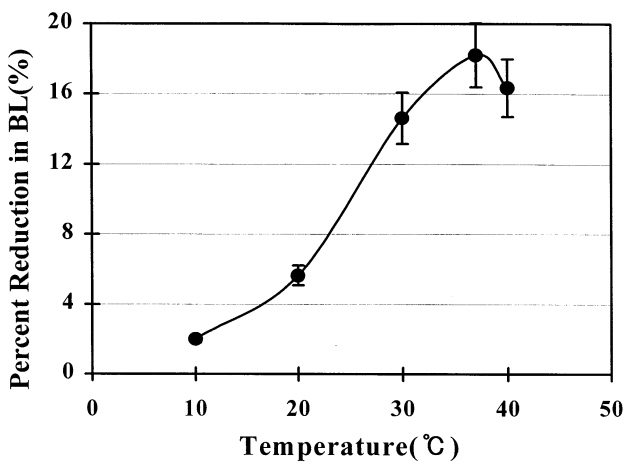

Fig. 5. Effect of temperature on the response ratio. Immobilized cell concentration was 1×10^9 cells/tube and a 2% benzene solution was injected. Temperatures are from 10 to 40°C.

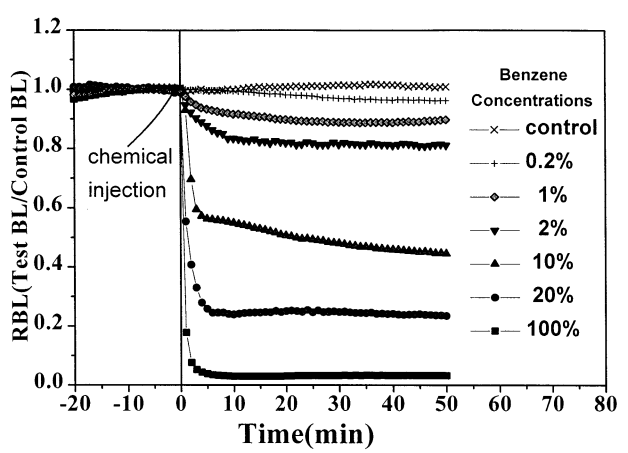

Fig. 6. Response to different benzene concentrations from 0.1 to 100%. Immobilized cell concentration was 1×10^9 cells/tube and temperature was maintained at 37°C.

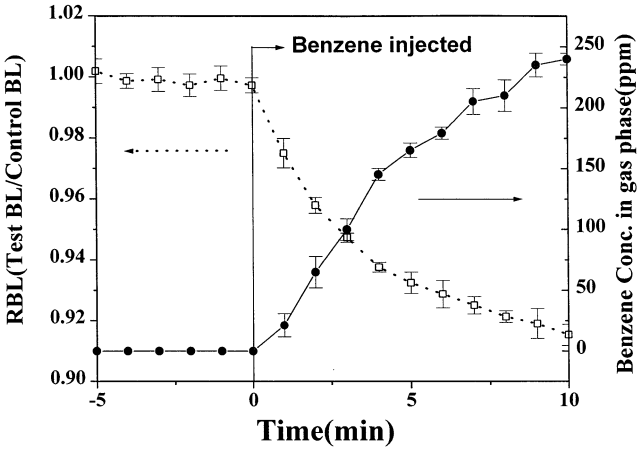

Fig. 7. Comparison between the measured benzene vapor concentration and BL profile. Immobilized cell concentration was 1×10^9 cells/tube, temperature was maintained at 37°C, and a 1% benzene solution was used. Benzene vapor concentrations were measured by gas chromatography (Hewlett Packard 5890 series).

medium, which was composed of 15 g/l Micro Agar (DUCHEFA, Netherlands) and 25 g/l LB broth (DIFCO, USA), was autoclaved and kept at 40°C in a water bath to keep this mixture from solidifying during the immobilization. Resuspended cells were mixed with 20 ml of the melted agar medium and then 100 μl of the cell mixture was immediately pipeted into each polypropylene test tube followed by agar solidification at room temperature (Fig. 1).

The tubes purchased from Turner Design (USA) were cut to a length of 10 mm (outer diameter, 7.5 mm) and then sterilized by autoclaving. The tubes containing the immobilized cells were sealed in a 1.5 ml centrifuge tubes and stored at 4°C. The concentration of the immobilized cells were determined by the volume of the cell culture and the optical densities (0.1 unit of $OD_{600} = 1 \times 10^8$ cells/ml) measured by a spectrophotometer (Perkin Elmer, USA). The cells were grown to nearly the same concentration with an optical density (OD_{600}) of about 4.4 whenever this preparation was performed. To immobilize different concentrations of cells in the tubes, 0.5, 5, 50, 100 and 365 ml cell cultures were centrifuged respectively and followed the same procedure above. The concentrations of the cells in the tubes were 1×10^7, 1×10^8, 1×10^9, 2×10^9, and 7.3×10^9 cells/tube.

2.3. Instrumentation, system and measurements

The biosensor kit in which the bioluminescent bacteria were immobilized was attached to the end side of a fiber optic light probe connected to a highly sensitive luminometer (Model 20c, Turner Design, CA) to measure the light output (bioluminescence) constitutively produced from the cells (Fig. 1). The luminometer was linked to a computer through a RS232 serial connection for the purpose of real time data acquisition. The test chamber was a 100 ml stainless steel cylinder with a water jacket to maintain a constant temperature using a thermostatic water bath (VWR Scientific, USA). The biosensor kit with the fiber optic probe was connected to an upside port in the chamber by black rubber tubing so as to prevent the leakage of the bioluminescence or the entering of ambient light. After a steady-state bioluminescence level (BL) was reached, 3 ml of the benzene solution was injected into the reactor through the syringe port to give various concentrations of vaporized benzene in the test chamber (Fig. 2) and the BL were measured every minute. In order to determine the real vapor concentration of benzene, 50 μl vapor samples were taken using gas tight syringes (Hamilton, USA) from the test chamber every minute, and injected into the septa port of a gas chromatograph (Hewlett Packard 5890 series, USA). An Ultra 1 capillary column and FID detector were used for the analysis.

3. Results and discussion

Expression of the *lux* genes from the *lac* promoter resulted in constitutive light emission (bioluminescence) by *E. coli* RFM443 carrying the recombinant *lux* plasmid. The bioluminescence level is expected to decrease with increasing inhibition on the metabolism of cells by toxic chemicals. Benzene, as an example gaseous chemical, was injected into the test chamber through the syringe port, shown in Fig. 2. The concentration of benzene was varied in a mixture with oleic acid. Oleic acid, known as a non-volatile compound (vapor pressure = 1 mmHg at 25°C), easily solubilizes benzene and was found to be non-toxic to the cells. Pure oleic acid injected into the chamber did not effect the bioluminescence level by itself (data not shown). Here, it can be assumed that only benzene is evaporated from the surface of liquid mixture. Constitutive light emissions from the recombinant *E. coli* were measured, through the fiber optic probe connection with the luminometer, every minute using a data acquisition system.

3.1. Prolonged steady-state bioluminescence

The cell immobilized tubes were stored in the refrigerator at 4°C. Since the centrifuge tubes are air tight, there was no free exchange of oxygen, thus lowering the growth rate even more. These biosensors were taken out from centrifuge tube and attached to the fiber optic probe in the chamber, which was maintained at 37°C, and immediately the BL was measured. Therefore, the abrupt exposure to oxygen and a temperature change would require some time for the cells to adapt to the

new conditions. Fig. 3 shows that the bioluminescence levels (BL) fluctuated, initially, for about 40 min during the adaptation time and then reached a uniform BL. The steady state BL level remained fairly constant for the next 200 min.

The lack of an increase in bioluminescence may be thought of as due to the low growth rate of the bacteria in the matrix, since the cells were immobilized during their early stationary phase. In fact, the cells were found to have been in a lag phase of growth for more than 150 min after immobilization and storage. This 3 h steady state is more than enough time to detect the toxicity of a gas because of the rapid detection time of the unit. Nearly the same amount of bacterial cells could be immobilized in each transparent polypropylene (PP) tubes with agar medium, therefore, the constitutive light emission would be the same in each PP tube (data not shown). This constant light level allows the comparison between the control BL without chemical injection and test BL that is repressed by toxic chemicals, therefore toxicity is observed using the relative bioluminescence (RBL = test BL/control BL), where

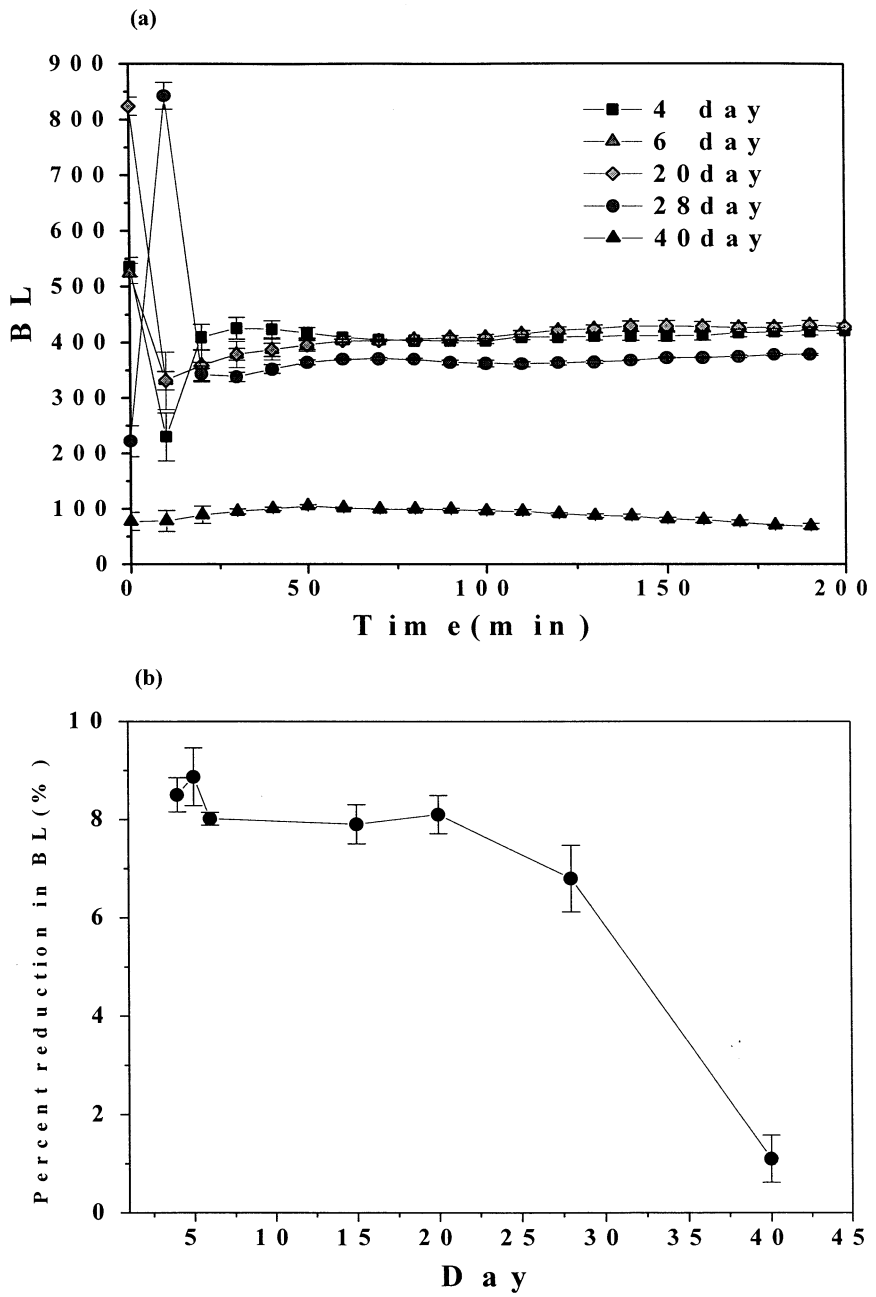

Fig. 8. (a) Cell stability (Data not shown for 5 and 15 day samples for clarity) and (b) response during long-term storage; cells were stored at 4°C and tested at 37°C; immobilized cell concentration was 1×10^8 cells/tube; 1% benzene solution was injected.

the control BL represents a steady-state BL without benzene injection.

3.2. Effects of the immobilized cell concentration

The effect the cell concentration has on the control BL of the immobilized bacteria was examined to obtain the optimum cell concentration showing the highest BL. Fig. 3 shows that the BLs for five different concentrations of immobilized cells were in an adjustment period for 40 min and then reached a steady-state. However, the steady-state BL levels were different for each of the cases. The steady-state BL values increased up to 1×10^9 cells/tube (case 3) and decreased when over 2×10^9 cells/tube (case 4). The declination of the steady-state BL may be related to the interference of metabolic activity between the immobilized cells because of high populations. Oxygen diffusion limitation may be a major factor in determining the BL, since oxygen is a necessary component in the bioluminescent reaction and as the cell density increases, a greater oxygen limitation within the matrix might occur. This would also explain the greater bioluminescence seen in case 5 as a greater cell density at the surface of the agar, thereby upsetting the diffusion limitation, would result in a greater bioluminescent output.

As a model toxic chemical in gas phase, benzene, in solution, was injected into the test chamber. A 2% benzene solution was injected and the % reduction ([steady-state BL − test BL]/[steady-state BL] × 100), representing the sensitivity of the biosensor, after 10 min was compared for the different concentrations of immobilized cells (Fig. 4). The higher the concentration of cells, the higher the reduction. This fact may be explained by considering that there are a greater number of cells on the surface of the solid agar medium in contact with benzene vapor directly (see Fig. 1). However, this response trend reversed when the concentration of cells was greater than 2×10^9 cells/tube, and this fact may also be understood by considering that too many cells may inhibit the biological activity necessary for the response. Therefore, the maximum response ratio was found to be when the concentration of the cells was between 1×10^9 and 2×10^9 cells/tube.

3.3. Effect of temperature

Microorganisms and their bioluminescent emission are sensitive to changes in temperature. Its influence on the BL of the immobilized cells was investigated for temperatures between 10 and 40°C. The maximum % reduction for a 2% benzene solution was found to be at 37°C (Fig. 5), which was thus defined as the optimum temperature for the growth of the strain used. Actually, the pLITE2 series strains were found to be most active at 37°C (Marincs and White, 1994). In addition, liquid benzene could be more easily vaporized at this higher temperature. However, the % reduction dropped when the temperature was above 37°C. Metabolism of the bacteria and activity of luciferase in the agar might be interrelated and inactivated by heat shock. Fig. 5 also shows that the activity of bacteria at lower temperatures abruptly drops, with the response at 10 and 20°C only 11 and 31% of the maximum reduction, respectively.

3.4. Dose dependent response for benzene vapor

Fig. 6 shows response curves of the gas toxicity biosensor to different concentrations of benzene solutions (0.1–100 volume %). After the BL reached a steady state, benzene solutions were injected. BL values decreased rapidly within 10 min after injection of the benzene solution into the test chamber, and then leveled off at a new steady state. As higher concentrations of benzene were injected, the response time, defined as the period of time for the new steady state BL to be reached, shortens and the initial declination slope also steepens. The new steady state after benzene injection still show some degree of light emission, since cells that do not have contact with gaseous benzene are still alive or exposure is low enough that the cells can overcome the toxic effect and metabolic inhibition by gaseous benzene. However, when purely benzene was injected, the BL dropped down to nearly zero, due to cell death brought on by the severe toxic effect of benzene.

Analysis of real benzene vapor concentrations in the test chamber was also conducted by using a gas chromatograph, and the corresponding concentrations of vapor benzene for a 1% benzene solution are shown in Fig. 7. As shown in Fig. 7, the RBL profile is well matched to the benzene vapor concentration measured during this experiment. It was found that the minimum detectable liquid benzene concentration was 0.2%, or approximately 48 ppm, in the gas phase from this analysis.

4. Discussion

The activity of bacteria may be affected by several factors such as temperature, immobilized cell mass, storage conditions, and so forth. The optimum conditions for the active sensing need to be maintained during the period of detection. Temperature is one of most important factors, and usually cells are active in the range of 30–37°C. Therefore, this biosensor may be faced with some problems when used in cold weather or environments, but if the biosensor kits are attached to the sensing apparatus and the air pumped in was kept warm, cells could manage to detect toxicity even in a cold gas phase. After immobilization, cells may slowly

grow in the agar medium and then reach the death phase, causing loss of activity, which is another problem for the long-term usage. We stored the cell matrix in the refrigerator in microcentrifuge tubes at 4°C, thus keeping the growth of the cells very slow, allowing storage for an extended time, of up to one month with a nearly constant response (Fig. 8a and b). Thus, this biosensor kit would be useful as a disposable component of a biosensor apparatus, whenever measurement may need to be done.

The minimum detectable concentration of benzene vapor was 0.2 volume %, which was approximately equal to 48 ppm of benzene vapor according to GC analysis. The study on the source identification for volatile organic compounds in chemical plants of Korea reported that VOC concentrations were 78.8–196.9 ppm in agricultural chemical factories, 103.8 ppm in epoxy factories, 3.2–63.7 ppm in dye factories, and so on, as measured by sample collection for 10 min (Lee et al., 1999). Therefore it is reasonable that this bioluminescent biosensor can detect toxicity in the air when exposed within the chemical plants. However, a more sensitive biosensor which is able to detect toxicity below the regulation levels is needed. The main barrier against such a highly sensitive reaction in our system may be the diffusion limitation of gas into the agar, the immobilization matrix. Adopting small glass beads may enhance the porosity of solid agar and, hence, facilitate gas penetration through the matrix. Such advanced immobilization steps would improve the sensitivity.

In this study, we used recombinant bacteria expressing bacterial bioluminescence constitutively under the *lac* promoter and so growth and metabolic inhibition occurs when cells are exposed to toxic gaseous chemicals. Therefore, classification of toxicity or its mechanism using this strain is nearly impossible. However, stress or chemical specific promoters induced by particular chemicals allowing the expression of the *lux* gene would be more advantageous for the detection of specific toxic chemicals. Finally, considering the cost for producing these types of biosensors and their ease of operation, future commercialization may be possible.

5. Conclusions

A gas toxicity biosensor using immobilized bioluminescent bacteria and a fiber optic probe was successfully developed. Benzene was selected as a sample toxic gaseous chemical to show the response of toxicity to the cells. The steady-state BL was maintained for about 200 min after the biosensor was activated by introduction into the measurement conditions. The response, which took only 10 min, demonstrates the rapid detection of gas toxicity by the biosensor. This biosensor was able to detect the toxicity of benzene gas reproducibly for different concentrations of benzene vapor. The minimum detection level was 0.2% liquid volume, or nearly 48 ppm of benzene in vapor phase. The small size of the biosensor kit, in which the bioluminescent *Escherichia coli* strain carrying plasmid pLITE2 was immobilized with agar medium, was found to be very advantageous because it could be applied as a portable biosensor.

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