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Immobilized Luminescent Cell-based Flow Through Monitoring of Environmental Pollutants

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A new sensing system based on the immobilization of luminescent bacteria, *photobacterium phosphoreum*, was proposed for continuous real-time monitoring of pollutants. The response curves demonstrate that *Photobacterium phosphoreum* immobilized on the strontium alginate were very sensitive to seven reference chemicals used. The significant inhibitory concentrations for bioluminescence emission were 5 ppm for $Pb(NO_3)_2$, $NiCl_2$, $CdCl_2$, 50 ppm for $NaASO_2$, 0.1 ppm for $HgCl_2$, 0.5 ppm for pentachlorophenol and less than 5 ppm for SDS, respectively. The alginate mixed-cells (AMC) retained their luminescence during experimental period (29 days) under storage condition of -80°C. The variables affecting performance of continuous flow through monitoring (CFTM) was optimized in order to ensure stability and efficiency. The flow through cell with strontium-alginate immobilized luminescent bacteria was tested with salicylate and 4-nitrophenol. A rapid response of luminescence was recorded by time drive mode in bioluminescence spectrometer after exposure to both toxicants.

Rapid and continuous detection of environmental contaminants is important for ensuring a high level of protection for the environment and public health. Although physicochemical methods have traditionally been employed for contaminants analysis, the coupling of biochemical reactions with optical biosensor for environmental monitoring of industrial pollutants such as toxic metals and chemicals has received intensive attention because of rapid response, low costs and improved reproducibility.

Many kinds of luminescent enzymes and microorganisms were used for analysis and detection. These included isolated luciferase for chemical assay (ATP) (4), BOD determination (6), and monitoring of metals and solvents (8, 14, 16, 17). An example of such methods for environmental monitoring is the measurement of metabolic activities by loss of bioluminescence from the marine microorganism *Photobacterium phosphoreum* involving the application of genetic manipulation with specific bioluminescent reporter functions (3).

Most biosensors for detection of chemi- and bioluminescence reactions used enzyme (5) luciferase as receptors of recognition of analytes and no methods based on flow through optical sensors with bioluminescent bacteria have so far been reported.

Generally, luminescent measurements were performed using discontinuous systems in which assay cuvettes containing enzymes and suitable reagents were disposed in batch luminometer. Thus, it was of interest to develop a continuous real-time monitoring of pollutant by activity potential of *P. phosphoreum*. The objective of this work was the development of a flow through optical sensor with immobilized luminescent cells for continuous and on-line monitoring of pollutants in environment.

Recently, the bioluminescent reporter bacteria, *Pseudomonas fluorescence* HK44, was immobilized onto the surface of an optical light guide by using alginate for on-line monitoring of naphthalene and salicylate bioavailability and microbial catabolic activity in waste stream (10). The immobilized bacterium was fully functional in strontium alginate providing a stronger matrix than calcium alginate. In this report, the *P. phosphoreum* was investigated to know whether cells immobilized in strontium alginate show a correlation between toxic pollutants and bioluminescence emission. For the application in the field, studies were also conducted to understand how immobilization affects bacterial activity and maintenance of bioluminescence when stored at appropriate temperature.

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Although most experiments were performed to determine toxicant concentration that would depress bioluminescence noticeably, the development of real-time detection method is addressing the qualitative, continuous interpretation of its results over a wide toxicity range.

MATERIALS AND METHODS

Bacterial Strain, Media and Growth Condition

The luminescent bacteria used was *Photobacterium phosphoreum* grown on NaCl medium at 18~20°C in a rotary shaker at 180 rpm for 24 h to an $O.D._{660}$ of 1.5~2.0. The inoculation was at between 10 : 1 and 20 : 1 dilution. The composition of NaCl medium is 12.5 g of nutrient broth No. 2, 5 g yeast nitrogen base without amino acids, 3 ml of glycerol, 25 g NaCl in 1 liter of distilled water. The pH was adjusted to 7.0 with 100 ml of a 50 mM potassium phosphate buffer prior to autoclaving.

Bacterial Reagent

A 200 ml portion of the culture grown at an optical density at 660 nm of 1.5~2.0 was centrifuged at 14,000 rpm for 20 min. The culture was washed once with 0.9% [w/v] NaCl solution and the pellet was resuspended in a 12.5 ml of sterile 0.9% NaCl solution. The optical density was checked and the culture was diluted to give a final concentration of approximately 2.0~2.5 at 660 nm. A 12.5 ml of the cell suspension was mixed with 25 ml of sterile physiological saline (0.9% [w/v] NaCl) and 7.5 ml of sterile glycerol as a cryoprotectant. For immobilization studies, 25 ml of a sterile alginate solution (3.5% [w/v] in 0.9% NaCl) was mixed thoroughly with cell suspension instead of 25 ml of 0.9% NaCl. The 20 ml ampule containing a 3 ml aliquot of cell mixture was stored at 4°C and -80°C.

Reactivation of Bacterial Reagent

The bacterial reagent stored at -80°C was thawed as quickly as possible by immersing the ampule in warm water. The thawed reagent was incubated at room temperature for 1~1.5 h for use in the bioluminescence measurement. For immobilized cells, the strontium alginate beads were made immediately after thawing and stirred in a 0.9% NaCl solution containing 0.1 M $SrCl_2$ for 1~1.5 h at room temperature before use. The thawing procedure was not necessary for reagents kept in a refrigerator at 4°C.

Immobilization of Cells

The strontium alginate was used as a strong matrix (9). A 10 ml of the cell matrix containing alginate and glycerol was passed dropwise through a syringe with a 10 gauge hypodermic needle into a 50 ml of stirred, sterile 0.1 M $SrCl_2$ solution. The beads that formed were slowly stirred (50~60 rpm) for 1~1.5 h at room temperature to harden and stabilize the strontium-alginate matrix. A cuvette (3 ml) was filled with beads obtained for bioluminescence measurement. The flow cell was prepared by injecting a 1.5 ml of cell-alginate mixture inside the flow-through cuvette containing 0.1 M $SrCl_2$ solution. The cuvette was then immersed in 20 ml of 0.1 M $SrCl_2$ solution for 1~1.5 h for further hardening and stabilization of bioluminescence emission.

Measurement of Bioluminescence

For measurement of the bioluminescence emitted by *P. phosphoreum* culture, the cuvette was localized inside the luminescence spectrometer chamber (LS-50 Luminescence Spectrometer, Perkin Elmer Pty. Ltd) fitted with total emission accessary. The instrumental parameters used were 520 nm of excitation with slit width 10 μm and 4810 nm of emission with slit width 10~20 μm. A calibration of light intensity was conducted by changing gate time from 80 to 180 sec. The cycle time was fixed at 200 sec throughout the measurement. The bioluminescence was integrated for 10 sec from 300 to 700 nm when using the read mode.

Flow Through Cell Design and Monitoring

The apparatus consisted of the luminescence spectrometer (described above), and a flow through rectangular cell with 10 mm path length (Starna Pty. Ltd. Australia) designed for fluorimeter. As shown in Fig. 1, the light emitted by a luminescence spectrometer in a fitted light-tight chamber. Both maintenance medium and pollutant solution contained 0.1 M $SrCl_2$ and nutrient components used in flask culture. The known amounts of each salicyate and 4-nitrophenol were used as the example toxicants. Feeding of fresh medium and toxicant solution through the cuvette was conducted with peristaltic pump. During the flow of the fresh medium, pollutant stream solution was pumped from the pollutant reservoir for 1~2 min resulting in a pertubation.

Data were recorded on a personal computer supplied

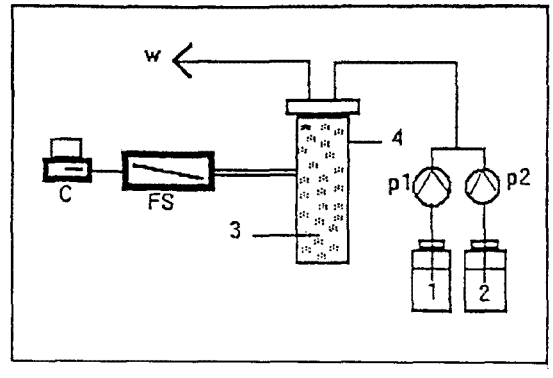

Fig. 1. Schematic diagram of the continuous flow through cell containing strontium alginate immobilized *Photobacterium phosphoreum*.

1, medium reservior; 2, pollutant reservior; 3, immobilized cells; 4, flow through cell; C, computer; FS, fluorescence spectrometer; P1 and P2, peristaltic pump.

with spectrometer. A change in bioluminescence intensity was monitored by determining the difference between the initial and the final intensity with or without analytes using time drive mode. In this case, the bioluminescence measurement system was conducted to integrate light emission from the cells for 10 sec to provide uniform light intensity. Data were obtained by taking an average measurement of duplicate samples.

RESULTS AND DISCUSSION

Seven toxic chemicals were chosen as reference which included five metals, $Pb(NO_3)_2$, $NaAsO_2$, $NiCl_2$, $CdCl_2$, and $HgCl_2$, and SDS and pentachlorophenol to evaluate response kinetics of immobilized cells. Several outcomes obtained from these tests were shown in Fig. 2. This poor light stability could be caused by the fact that cells had been stored at -80°C for three weeks before being used for this experiment.

During storage, cellular supplies of components for light reaction might be reduced. Neverthless, the response curves demonstrate that immobilized cells are very sensitive to all the reference chemicals used at these concentrations. As far as the immobilized cells are concerned, the significant inhibition concentrations were 5 ppm for $Pb(NO_3)_2$, $NiCl_2$, $CdCl_2$, 50 ppm for $NaAsO_2$, 0.1 ppm for $HgCl_2$, 0.5 ppm for pentachlorophenol and less than 5 ppm for SDS. Increasing the concentrations of these chemicals and the time length of exposure generally resulted in greater inhibition of light. Concentration of lower than 0.1 ppm of pentachlorophenol stimulated light whereas 50 ppm $NaAsO_2$ was very toxic in the first hour or so of exposure after which further inhibition was not seen. $NiCl_2$ and $CdCl_2$ at the concentration of 10 ppm or lower stimulated bioluminescence. $HgCl_2$ and pentachlorophenol were the most toxic chemicals tested to the cells. It was also confirmed that the kinetics responded by immobilized cells is generally time and dose dependent: the more of the chemicals and the longer the exposure is the greater the inhibition will be except for $NaAsO_2$ while this trend is not as clear for free cells. In summary, the toxicant-sensitive immobilized cells are still valuable for the application for toxicant monitoring and their response to toxic substances may be more quantitative than that of free cells, one of the key factors for a defined biosensor.

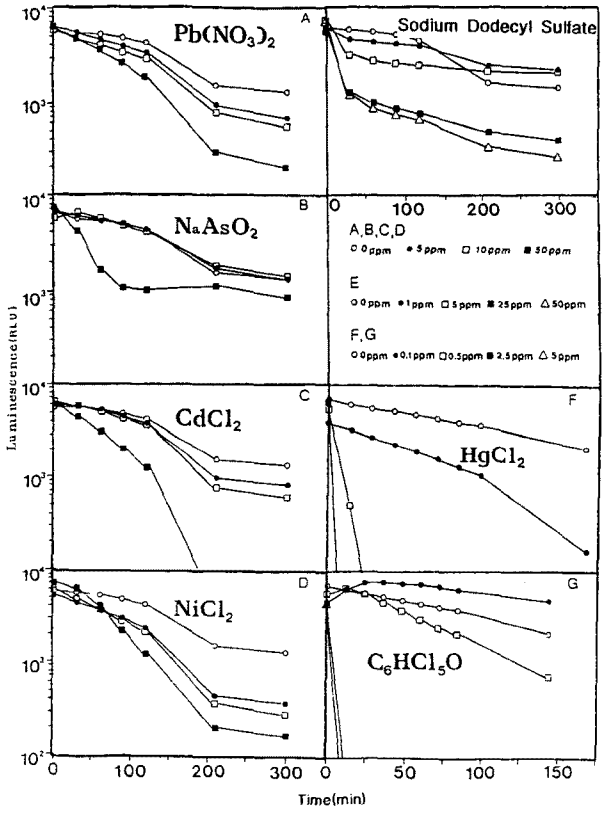

Fig. 2. Bioluminescence responses of immobilized *P. phosphoreum* at various concentrations of toxicants.

Effect of Storage Conditions

A long term experiment was performed to determine how storage time affects bioluminescence emission of both alginate free- and mixed- *P. phosphoreum*. For alginate free-cells (AFC), 25 ml of 0.9%[w/v] NaCl and 7.5 ml of sterile glycerol were mixed with 12.5 ml of bacterial suspension for storage. The bacterial reagent for immobilization was prepared with 25 ml of low viscosity alginate solution (3.5%, w/v, in 0.9% NaCl) and 7.5 ml of sterile glycerol to give same concentration microorganism. Both bacterial reagents were stored at 4°C and -80°C in ampules for 14 and 29 days, respectively, and ampules were taken regularly.

The stabilization time after thawing was described earlier in previous work (2). The data obtained (Fig. 3) showed that the alginate mixed-cells (AMC) for immobilization stored at -80°C considerably retained bioluminescence over the experimental period although the bioluminescence had diminished by about 30% one day after storage. The bioluminescence also remained almost constant over 14 days with AMC stored at 4°C. With alginate free-cells (AFC), although the bioluminescence decreased by about 55% after 25 day storage at -80°C, the constant bioluminescence was measured with cells stored at 4°C. During storage at both temperatures, the AMC reagent resulted in higher bioluminescence than AFC.

It is proposed that the stable maintenance of bioluminescence for AMC is probably due to protecting mechanism for biochemical activity of microorganisms during storage as well as thawing. Although the cell vi-

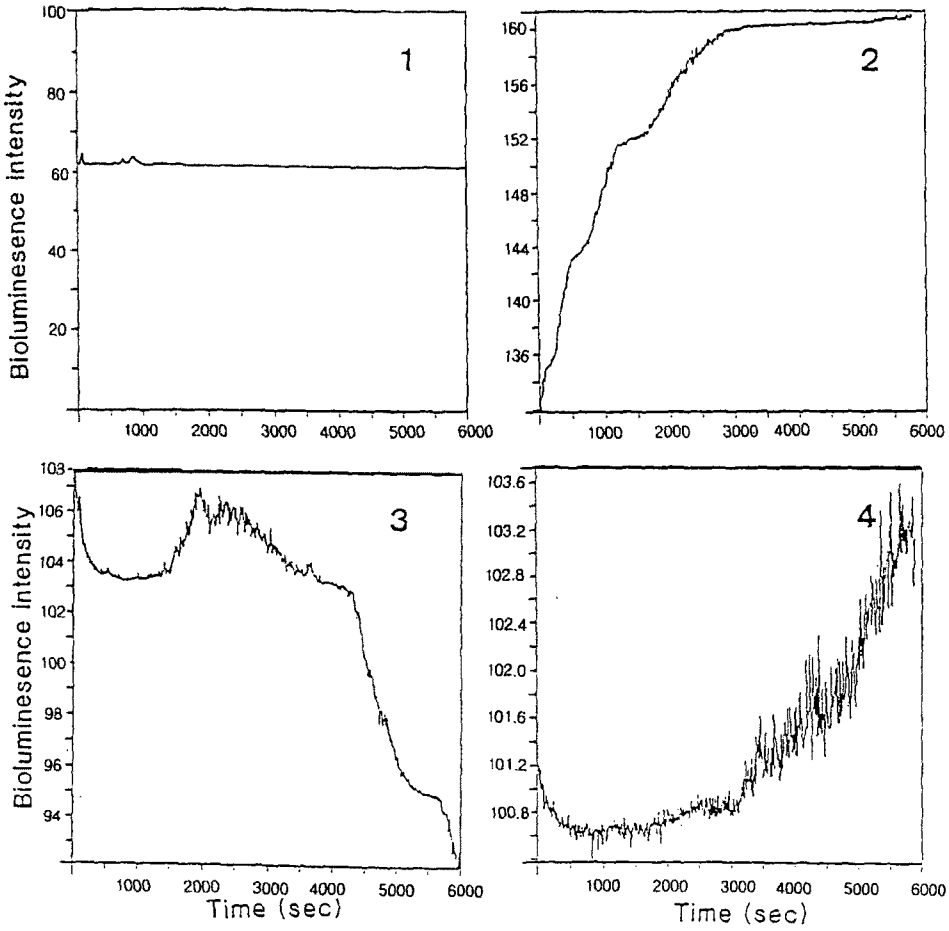

Fig. 3. Bioluminescence profiles of stock solution for the first 150 min.
(1,2) storage at 4°C; (3,4) storage at -80°C and (1,3), free cells; (2,4), immobilized cells. Bioluminescence intensity is represented by arbitrury unit.

ability was not measured in this work, it was reported that the level of metabolic activity in the immobilized cells was independent of cell viability (10). Furthermore, Fig. 3 shows how alginate affects the reactivation of bioluminescence emitting activity. The bioluminescence was measured for 150 min after warming-up and thawing of 4°C and -80°C stored cells, respectively.

The AFC stored at 4°C emitted a constant bioluminescence. However, the bioluminescence markedly decreased with AFC stored at -80°C. In contrast, an initial increase in luminescence followed by plateau was observed in the immobilized cells stored at both conditions. This result supports the bioluminescence of the immobilized cells that was significantly higher than that of the free-cells shown in Fig. 4. It is confirmed that the AMC retain their bioluminescence emitting activity during storage at -80°C providing a useful method to construct a continuous biosensing system with immobilized luminescent bacteria.

Application of Immobilized Cells

The flow through cell with immobilized *P. phosphoreum* was prepared as described in Materials and Methods. The variables affecting the performance of a continuous flow through monitoring (CFTM) was optimized in order to ensure stability and efficiency. Table 1 shows the range over which variables were studied and the optimal value found. Although the room temperature was used, the bioluminescence detecting chamber located with the flow cell was kept cool with cooling air.

The flow rate of toxicant solution through the cell influenced the performance of the biosensing system, since the toxicant - cell (enzyme) contact time was strongly dependent on this hydrodynamic variable. The flow rate of 25 ml/h was selected as optimal as it yielded the good sensing signal in a short residence time of 37 sec with injection time of 1~2 min. Higher flow rate resulted in inadequate development of process, whereas the lower values were too time-consuming for real time detection.

Various pH ranges were tested for maintenance medium.

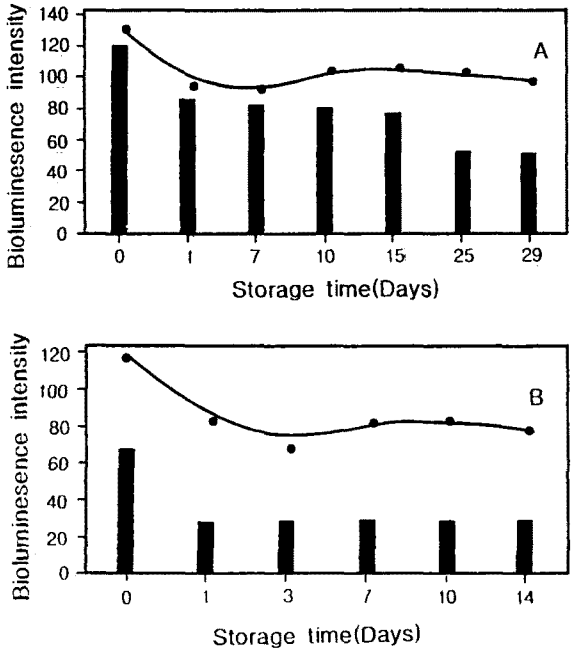

Fig. 4. Effect of storage time on bioluminescence of the alginate free- and alginate mixed cells.

For free cells, 25 ml of 0.9% NaCl and 7.5 ml of sterile glycerol were mixed with 12.5 ml of bacterial suspension for storage. The bacterial reagent for immobilization was prepared with 25 ml of low viscosity alginate solution (3.5 ml, w/v, in 0.9% NaCl). (A), storage at -80°C; (B), storage at 4°C. Bioluminescence intensity is represented by arbitrury unit. ■, free cell; ●, alginate mixed cell.

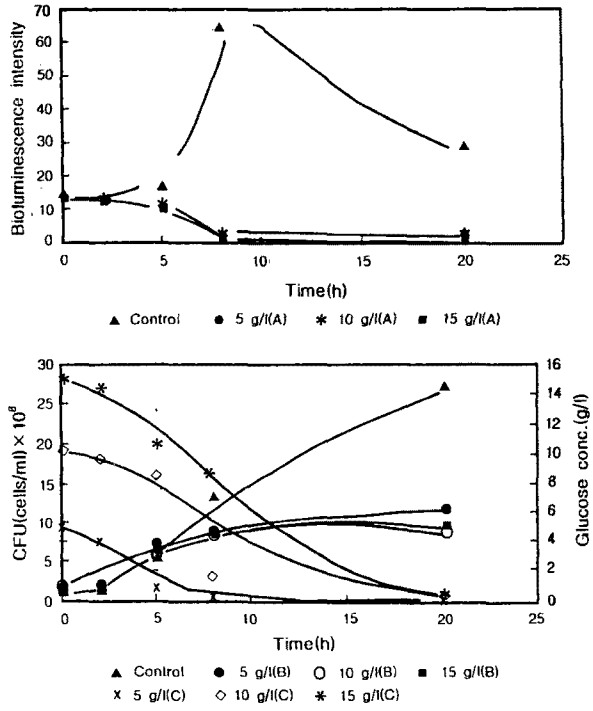

Fig. 5. The effect of glucose on the emission of bioluminescence and CFU with *Photobacterium phosphoreum*.

Shake flask culture was performed in medium containing 25 g/l of NaCl, 12.5 g/l of nutrient broth, 5 g/l of yeast extract, and 3 ml/l of glycerol at 18°C. (A), bioluminescence; (B), colony forming unit (CFU); (C), glucose concentration. Bioluminescence intensity is represented by arbitrury unit.

Although pH higher than 7.0 showed little or no effect on the bioluminescence emission with immobilized cells, pH 7.0 was adjusted with 100 ml of a 50 mM potassium phosphate buffer per liter. Stabilization time was 1.5~2 h after preparation of a flow through cell as discussed in the previous work (2).

Table 1. Optimization of variables for continuous flow system with immobilized *Photobacterium phosphoreum*.

Parameters	Ranges studied	Optimal conditions
Temperature	–	room temperature
Flow rate (ml/h)	8.3~35.0	25
Injection time (min)	1~5	1~2
pH range	2.5~10	7
Thawing temperature	–	18°C
Stabilization time after immobilization	1~2.5	2
Carbohydrates;		
glucose (g/l)	5~15	none
sucrose (g/l)	2~10	none
Inducer;		
thymine (μg/l)	100~300	none
homoserine-lactone (μg/l)	100~300	none
aldehyde (μg/l)	10~50	none

Induction of bioluminescence occurred at mid-log phase of cell growth and the increase of luminescent intensity was far more rapid than cell growth, but luminescence rapidly decreased after the culture had passed this point (as discussed in the previous work (2)). Although this might be due to the inner filter effect, some carbon sources or chemicals reported to be inducer for *Vibrio fisheri* were added to the culture media to try to keep extended expression of the *lux* operon. However, neither glucose nor sucrose were found to affect the maintenance of bioluminescence emission. Both carbohydrates showed inhibition on bioluminescence and growth of microorganism. For glucose concentrations of 5~15 g/l, the inhibition of bioluminescence was accompanied by a proportional inhibition of growth (Fig. 5). Sucrose also showed similar effect on bioluminescence and growth (data not shown).

This result was further confirmed by entrapped cells in a hollow fiber membrane. Microorganisms were entrapped between cartridge and membrane module, and a medium was flowed through the lumen at a dilution rate of 0.05 h^{-1}, thus no cells were removed. Fig. 6 clearly

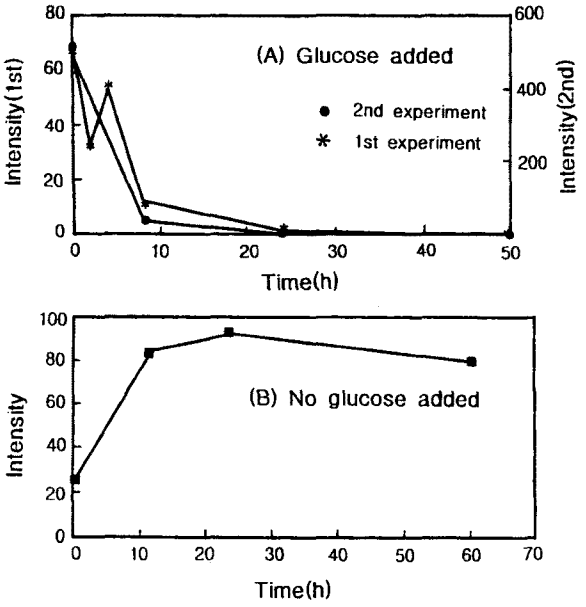

Fig. 6. Continuous process with entrapped cells in hollow fibre membrane.
(A), glucose (5 g/l) was added to feeding medium; (B), no glucose was added. Bioluminescence intensity is represented by arbitrury unit.

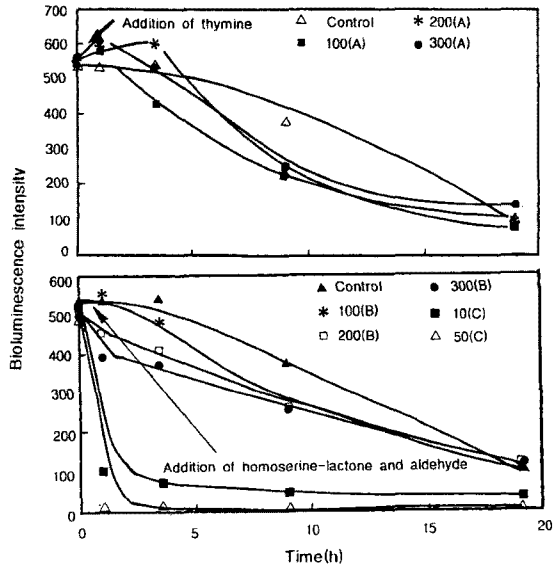

Fig. 7. The effect of thymine, homoserine-lactone and aldehyde on the bioluminescence emission with *Photobacterium phosphoreum.*
Cells were grown in shake flask for 27 h at 18°C before the addition of above chemicals and samples were collected 10 min after addition. (A), thymine (μg/l); (B), homoserine-lactone (μg/l); (C), aldehyde (μg/l). Bioluminescence intensity is represented by arbitrury unit.

shows that glucose (5 g/l) strongly inhibited luminescence emission, whereas bioluminescence was emitted stably in the case without glucose. Catabolite repression was also reported with *V. haveyi* and *V. fisheri* in which the bioluminescence emission was partially restored on addition of cAMP (1).

The effects of thymine, homoserine-lactone and aldehyde, which are known to be stimulator, autoinducer and substrate, respectively, were tested to know whether they maintain the luminescence at the time of diminishing of light emission.

The synthesis of thymine is inhibited by an antibiotic, sulfamethizole. This antibiotic directly inhibits dihydropteroate synthetase, an enzyme in the pathway leading to the formation of dihydrofolate and thymine in *P. phosphoreum* (18). Thymine was able to give some effect on the expression of the luciferase system on thymine-deficient medium. It is believed that thymine is a main compound for a bioluminescence reaction. The time-course for luminescence with and without added thymine are shown in Fig. 7A. The addition of 200 and 300 μg/l of thymine to the medium at 27 h culture stimulated luminescence emission to some extent, but not substantially.

Although N-acyl-L-honoserinelactone, which is synthesized dependently on autoinducer synthase gene *luxI*, is the autoinducer for population density-responsive induction of the luminescence operon (*luxICDABEG*) in *V. fisheri* (9), it was not attributed to the effect on the luminescence in *P. Phosphoreum* (Fig. 7B). The addition of aldehyde (11, 50 μl/l) to a culture medium showed even adverse effect on bioluminescence activity (Fig. 7C). Thus, none of such chemicals was added to the maintenance medium.

Real-time Monitoring with Flow Cell

Our results confirmed that bioluminescent strain of *P. phosphoreum* retained their emitting activity and response sensitivity when cells were stored under appropriate conditions followed by immobilization. Therefore, it was possible to immobilize the luminescent bacteria with strontium alginate to construct an real-time monitoring system.

The flow through cell sensor prepared was operated at continuous manner with pulse feeding of toxicant. The bioluminescence signal to a 4-nitrophenol (10 ppm) and salicylate (50 ppm) in the pollutant stream monitored with time drive mode is shown in Fig. 8. The response time of a sensor after exposure to the toxicants was between 4 and 6 min for 4-nitrophenol and salicylate, respectively. It is interesting to note that the bioluminescence emission recovered very quickly when the toxicant solution was switched to a maintenance medium providing substrates, nutrients at a constant rate for the immobilized bacteria. This can be explained by the fact that the toxicant such as phenol (in this work) or antibiotic, sulfamethizole (18), inhibits bioluminescence sensitively without lowering cell growth or cellular levels of luciferase, reduced flavin or aldehyde.

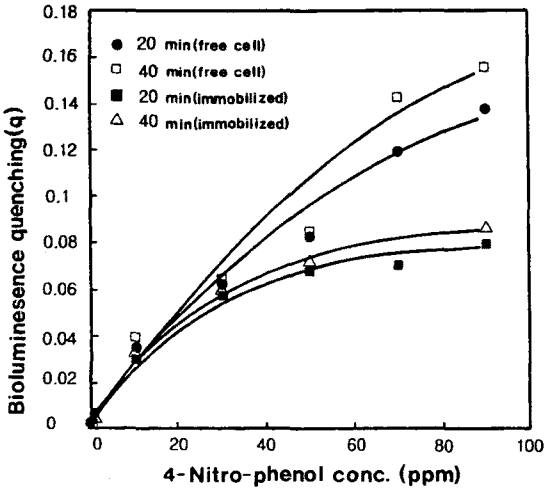

Fig. 8. Comparative quenching of bioluminescence by 4-nitrophenol for both free and immobilized cells.
Equal concentration to the free cells was contained in immobilized cell gel.

The luminescence intensity after a change from 4-nitrophenol or salicylate to maintenance medium was rapidly recovered and then slightly decreased. A number of factors could contribute to the decreasing bioluminescence including limited availability of luminescent reaction substrates such as O_2, aldehyde, ATP, or $FMNH_2$ and intrinsic stability of the luciferase enzyme. The remaining toxicant in the flow cell might also cause a decrease in bioluminescence.

These results show only qualitative response to pollutants. Further research is required to correlate the signal intensity with the actual concentrations measured by the analysis. In spite of that, rapid sensing signal with CFTM can be used for cost-effective system documentation or, under more sophisticated circumstances, to provide direct feedback for real time system control.

Whole living-cell biosensor has been reported for a number of compounds (13). Systems used for environmental pollutant monitoring were based on respirometric (7, 12) or calorimetric (15) measurement, thereby making use of an overall physiological response of an organism after exposure to a pollutant. One of the main advantage of the immobilized luminescent cell sensor over enzyme biosensor is high stability and maintenance of bioluminescence with storage, and the information on physiological and biological parameters that can be obtained. The flow cell with immobilized cells also allows sensor to monitor continuously at real time.

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