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Stabilization of Bioluminescence of Immobilized *Photobacterium phosphoreum* and Monitoring of Environmental Pollutants

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Stability of bioluminescence was investigated with *Photobacterium phosphoreum* immobilized on the strontium alginate in order to develope continuous real time monitoring of pollutants. The stability of bioluminescence emission was improved by prolonged aging time. The aging time of $\geq$40 min and the cell concentration of $\leq$0.6 of OD_{660} were selected for the immobilization of *P. phosphoreum* to give linearity between cell concentrations and bioluminescence intensity. In sensitivity tests using phenol, it was found that this compound quenched bioluminescence proportional to the concentration without lowering of cell growth. The lower value for maximum quenching (q_s) and higher dissociation constant (K_s) were observed with strontium-alginate immobilized cells compared to free cells. The response of bioluminescence to toxicants was evaluated with the immobilized luminescent bacteria. The sensitivity of the immobilized cells was found to be good in response to toxicants, 4-nitrophenol, salicylate and cadmium, when evaluated with a specific rate of bioluminescence quenching.

All kinds of luminescent enzymes and microorganisms were used for analysis and detection. These included isolated luciferase for chemical assay (ATP) (3), BOD determination (8), and monitoring of metals and solvents (12, 21, 23, 24). 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 (2).

The luciferase reaction in *P. phosphoreum* can be considered to be highly specific for $FMNH_2$, although related flavin derivatives can act on light emission but with a much lower efficiency (16). The biochemical and genetic charaterization of bacterial bioluminescence reaction has also revealed that a heterodimeric luciferase enzyme is encoded by the *luxA* and *luxB* genes (6, 18). The bioluminescence emission is generated by oxidation of $FMNH_2$ in the presence of molecular oxygen and of long-chain aldehyde (R-CHO). The synthesis of the aldehyde is catalyzed in an ATP and NADPH dependent manner by a multienzyme, fatty acid reductase complex comprising a reductase, a transferase, and a synthase encoded by the *luxC*, *luxD*, and *luxE* genes, respectively (17).

Most biosensors for detection of chemi- and bioluminescence reactions use enzyme, 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 investigation of stability of immobilized *P. phosphoreum* for 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 (7). The immobilized bacterium was fully functional in strontium alginate providing a stronger matrix than calcium alginate. In this report, the *P. phosphoreum* was

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investigated to know whether cells immobilized in strontium alginate show a correlation between toxic pollutants and bioluminescence emission.

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 was 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 broth ($O.D._{660}$=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 with O.D. 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 (7). 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 489 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.

RESULTS AND DISCUSSION

Factors Affecting Stability of Bioluminescence

In order to develop a suitable monitoring system, some preliminary experiments were carried out to ensure compatibility between development and stabilization of efficiency. A concentration of organism is an important factor for the immobilized cell biosensing system. It is expected that low concentration of cells emits low intensity of bioluminescence and, on the other hand, high number of organisms gives more intensive light reading.

The effect of cell concentration was investigated in a series of tests where multiple measurements were taken at various levels of organism concentrations. The *P. phosphoreum* was cultivated in the shake flask at 18~20°C, 180 rpm for 24 h throughout experiments.

Before neasurement of the bioluminescence, a 1~3 ml aliquot was taken from the shake flask and diluted with 2.5% NaCl solution. A 3 ml aliquot was taken into the cuvette from this diluted solution and then shaken for 5 sec with hand. The bioluminenscence was measured twice, and the average was recorded. Next sample was prepared at different dilution ratio with culture broth withdrawn from the shaker one by one giving an immediate measurement from the shaking condition of the culture. The bioluminescence was determined every 5 min for 20-min period at room temperature.

As Fig. 1A shows, the resulting plot indicates a lack of correlation between organism concentration, represented by OD_{660}, and bioluminescence intensity. In the first 0 and 5 min time, the light emission was proportional to the cell concentration and it was then decreased against concentration. However, a destinct linear relationship was eventually shown after 10 min. The specific rate of bioluminescence change for 20 min was determined as

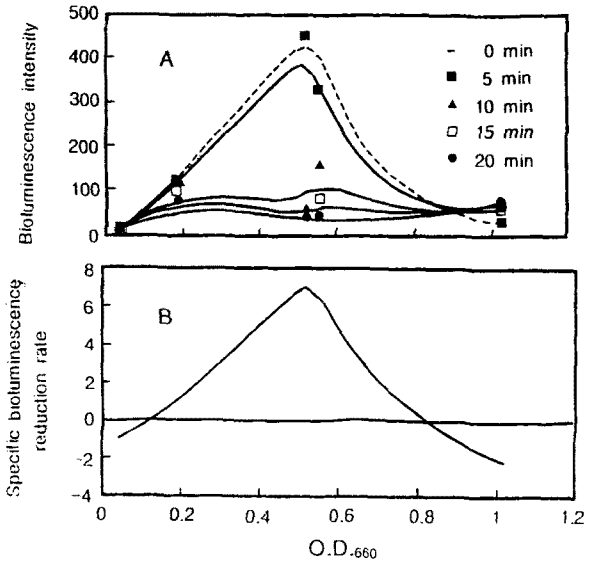

Fig. 1. The profile of bioluminescence vs. concentration of *Photobacterium phosphoreum*.
A 3 ml aliquot was taken from the shaken flask culture and diluted with 25% [w/v] NaCl solution at various ratios. Bioluminescence was measured every 5 min for 20-min period. The specific bioluminescence emission (μ) was also calculated from the difference between initial and final light intensity for 20 min. Bioluminescence intensity is represented by arbitrury unit.

$$\mu = (\ln L_2 - \ln L_1)/(t_2 - t_1) \quad (1)$$

where μ is the specific rate of bioluminescence change, t_1, t_2 are 0 time and 20 min, respectively. L_1 and L_2 are defined as bioluminescence intensity measured at 0 and 20 min. As plotted in Fig. 1B, the resulting variability appeared to be strongly related to the concentration of organism and aging of cells.

The effect of microorganism concentration on the bioluminescence was further studied with batch culture experiment. The samples taken from shake flask were diluted at 1 : 2 ratio to give OD_{660} range between 0 and 1.0. Fig. 2 shows the typical uncoupling between microorganism growth and bioluminescence emission. The maximum bioluminescence intensity was appeared at OD_{660} between 0.5 and 0.6. The light emission then decreased although the microorganisms were still growing. Such phenomena could be explained as the inner filter effect in which bioluminescent cultures not only emit light but also absorb and scatter it (11, 13, 28). While at low cell concentrations this effect causes a relatively small error of a few percent, at high density this error increases up to several hundred percent causing the measured light intensity in a complex, non-linear. In biochemical view, bacterial luciferase is known to be an inducible enzyme synthesised under a complicated control system. The enzyme synthesis in batch culture starts with accumulation

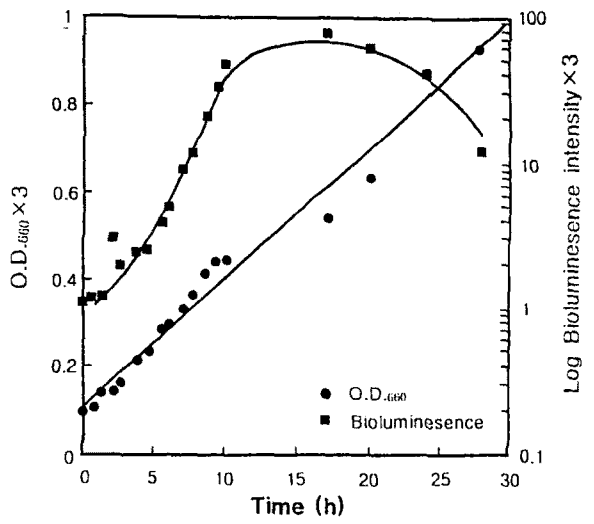

Fig. 2. Uncoupling of growth and bioluminescence emission.
Bioluminescence intensity is represented by arbitrury unit.

of a specific substance in a medium, an autoinducer (18, 19). It was reported that enzyme synthesis ceased and inactivation started when the culture reached a certain level of luminescence, though the bacteria were still growing (20).

A comparison of the results in Fig. 1 and 2 indicates that the both concentration of microorganisms and aging time may be the main factors affecting effeciency of immobilized cell system. This can also lead to the conclusion that an adequate interpretation of the bioluminescence signal is difficult without optimization of variables influencing the performance.

The aging time of 40 min was selected to investigate the effect of cell age on the stability of light emission, where the series of concentrations was prepared with dilution harvesting from the shake flask culture. The bioluminescence was measured at 5 min interval for 25 min, followed by the first measurement. Another experiment was conducted simultaneouesly with this procedure except immobilization of cells. Each concentration of free cells was mixed with alginate solution (3.5%[w/v] in 0.9%[w/v] NaCl solution) at 1 : 2 ratio. The luminescence emission responses show a linear function of cell concentration in both experiments (Fig. 3, 4) indicating that the stability could be improved by prolonged aging time. The average of counts also confirms the linear increase of bioluminescence emission with increasing cell concentration. Thus, the aging time of > 40 min and the cell concentrations < 0.6 of OD_{660} were used for the immobilization studies to avoid any source of error in bioluminescence measurement resulting from the so-called aging time or inner filter effect.

Quenching of Bioluminescence with Toxicant

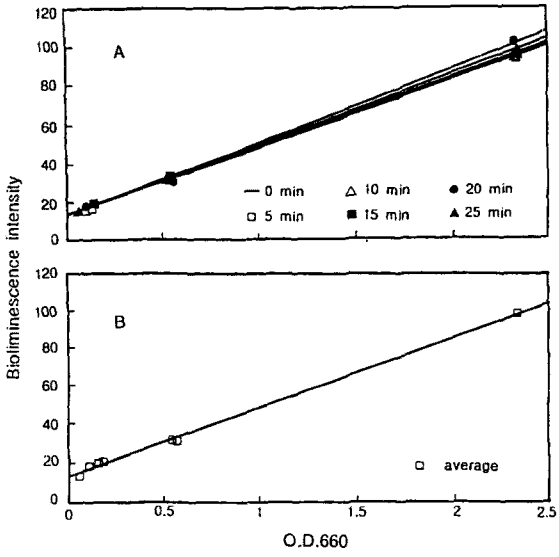

Fig. 3. Bioluminescence profile vs. concentration of free *Photobacterium phosphoreum* cells.
1.0 ml aliquot of cell suspension was mixed with 2.0 ml of 0.9% (w/v) NaCl solution. The bioluminescence measurement was started at 40 min after harvesting from shake flask culture. (A) A bioluminescence vs. OD_{660} measured every 5 min for 25 min. (B) Correlation curve between average bioluminescence of 6 units and OD_{660} for 25 min. Bioluminescence intensity is represented by arbitrury unit.

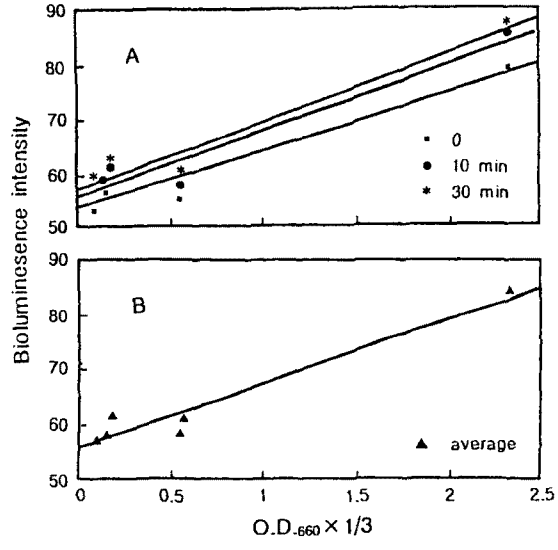

Fig. 4. Bioluminescence profile vs. concentration of immobilized *Photobacterium phosphoreum.*
Each concentration of free cells was mixed with alginate solution (3.5%, w/v, in 0.9% NaCl) at 1 : 3 ratio. (A) A bioluminescence vs. OD_{660} measured at 40 min after harvesting from the shake flask culture with 10 min interval. (B) Correlation curve between average bioluminscence of 3 counts for 30 min and OD_{660}. Bioluminescence intensity is represented by arbitrury unit.

There are many reports about biochemical and genetic research with the bioluminescent bacteria that have been

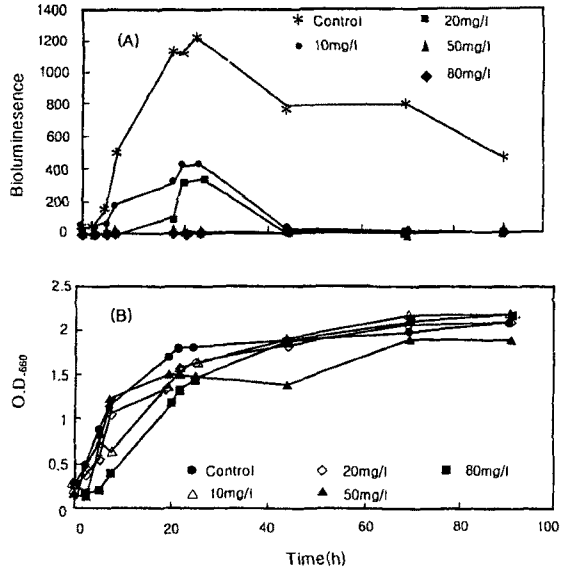

Fig. 5. Biolumonescence quenching caused by phenol.
Photobacterium phosphoreum was grown at 18°C and various concentrations of phenol were added to culture flasks at time zero. (A) Bioluminescence. (B) Cell growth. Bioluminescence intensity is represented by arbitrury unit.

suggested as a bioassay reagents for determining toxicity in aquatic environment. The bioassay is based on monitoring change in natural light emission (i.e., quenching) from the bioluminescent bacteria when challenged with a toxic substance. However, the biochemical explanation of quenching mechanism is quite complex and mostly unknown. Fig. 5 shows the bioluminescence profile with various concentrations of phenol. The phenol was selected as a model toxicant since it is a basic compound that can be substituted with functional groups and comparatively toxic (i.e., $\log EC_{50}$=2.63 μmol/l with *P. phosphorem*) (9), but much less genotoxic than 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) or many polycyclic aromatic hydrocarbons (1). The bioluminescence was slightly quenched with 10 mg/l of phenol, while at high concentration (> 80 mg/l), almost no bioluminescence was detected throughout the culture period. It is also interesting to note that the phenol did not inhibit the growth of cells within a cencentration range tested assuming a enzymatic mechanism of the organism's response to phenol. This phenomenon was also reported with an antibiotic, sulfamethizole; luminescence was strongly inhibited at a concentration where growth was not (25).

The relation between quencher (phenol) concentration and light intensity in Fig. 5 resemble a so-called dynamic quenching process, where fluorescence is quenched by virtue of an excited state interaction with the analyte (quencher) (27). The initital intensity remains constant, but the decay is faster with quencher so that

lifetime is reduced. Theoretically, the luminescence intensity varies with quencher as follows:

$$I_o = I/(1 + K_s[Q]) \quad (2)$$

where I_o and I reflect the intensity of luminescence in the absence and presence of quencher, respectively. K_s is constant. Equation can be rearranged to give

$$I_o/I = 1 + K_s[Q] \quad (3)$$

which is the equation for a static luminescence quenching that does not influence the lifetime of the light, but rather its emission intensity.

For the dynamic luminescence quenching, the question can be written as

$$I_o/I = \tau_o/\tau = 1 + K_d[Q] = 1 + k_q \cdot \tau_o[Q] \quad (4)$$

with K_d being the overall quenching constant and τ_o, τ, the lifetimes in the absence and presence of quencher (phenol), respectively. K_d can be shown to be the product of the lifetime τ_o of the luminescence in the absence of quencher and the biomolecular quenching constant k_q:

$$K_d = k_q \cdot \tau_o \quad (5)$$

From the equations 4 and 5, it is expected that the bioluminescence can decrease with quencher concentration and increasing k_q. The k_q is diffusion-dependent, so that the quenching efficiency can be controlled to some extent by chemical properties like water solubility and octanol/water partition coefficient (4, 9).

The phenol is not bioavailable compound with *P. phosphoreum* and, hence, the plot of I_o/I vs [Q] was drawn from the data obtained at 21 h culture which showed good responses to the phenol (Fig. 6). A linearity between I_o/I and [Q] is not perfect probably due to the high concentration of phenol. The solution dielectric constant and ionic strength can also influence the deviation when the quenchers are ions (5, 10). The 1/Kd estimated as 0.58 mg/l from the Fig. 6, phenol concentration when I_o/I=2, half the maximal luminescence, is very low indicating a good distribution of phenol to hydrophobic areas of the microorganism.

There are many reasons why bioluminescence is depressed by a particular compound including changes in membrane potential or interference with RNA or DNA functions. The result observed with phenol indicates that the quenching reaction may be based on a enzyme reaction such as inhibition of dihydropteroate synthase, an enzyme in the pathway leading to the function of dihydrofolate and the synthsis of thymine (25). The rapid quenching reaction with phenol or its derivatives is an advantage for developing continuous real-time monitoring system (CRMS).

Evaluation of Immobilized Cells

The toxicity of a chemical is one effect of the total biochemical interaction of the substance with the microorganism. In order to enter a cell, a chemical must diffuse through the immobilization matrix, i.e., alginate, and cell's lipid membrane. 4-nitrophenol and salicylate were selected to evaluate toxicity with immobilized cells

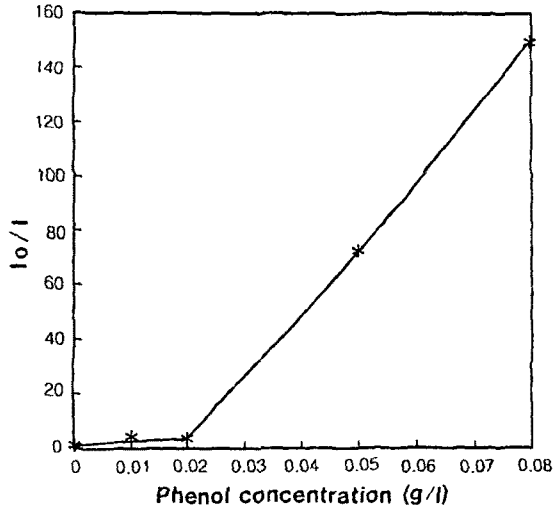

Fig. 6. The relationship between phenol concentration as a quencher and bioluminescence intensity (I_o/I) measured at 21 h culture of *Photobacterium phosphoreum*.
Bioluminescence intensity is represented by arbitrury unit.

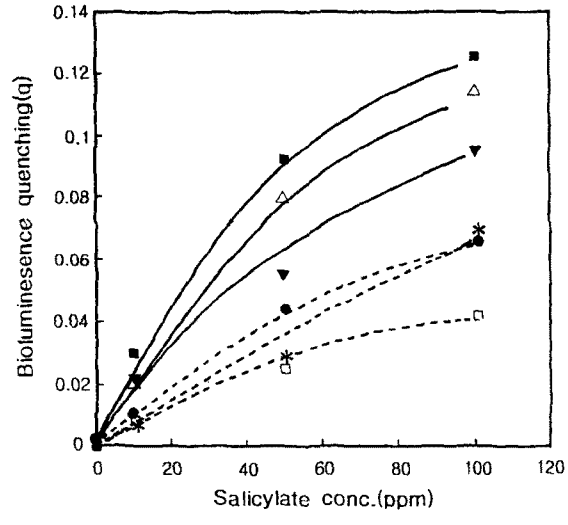

Fig. 7. Comparative quenching of bioluminescence by salicylate for both free and immobilized cells.
Equal concentration to the free cells was contained in immobilized cell gel. —▼—, 10 min (free cell); —□—, 10 min (immobilized); —△—, 20 min (free cell); —*—, 20 min (immobilized); —■—, 50 min (free cell); —●—, 50 min (immobilized).

since they have been reported to show higher n-octanol/water partition coefficient giving more sensitive toxic effect than phenol. The response of both free and immobilized cells to various concentrations of 4-nitrophenol and salicylate was determined by comparing bioluminescence intensity with toxicant, L_s, to that of control, L_o and calculating as

$$q = (L_o - L_s)/L_o \quad (6)$$

The value of quenching was then plotted against the toxicant concentrations giving a similarity to typical curves representing enzyme-reaction kinetics (Fig. 7, 8). The quenching kinetics with other aromatic hydrocarbons were reported to be based on the enzymatic reaction (22, 14).

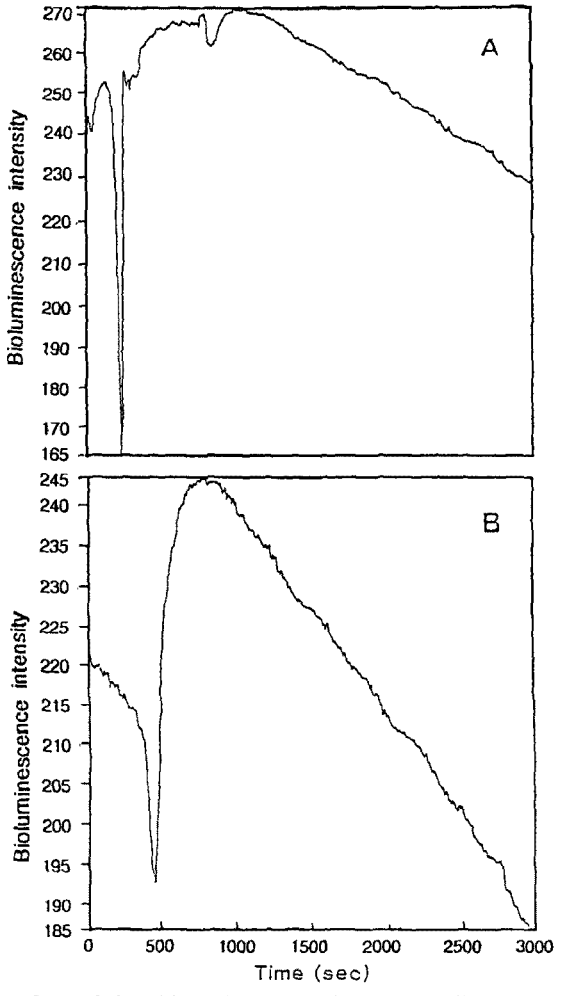

Fig. 8. Real time bioluminescence signal responding to (A) 4-nitrophenol (10 ppm) and (B) salicylate (50 ppm) continuous flow through optic sensor with immobilized *Photobacterium phosphoreum*.
Flow rates of both substrates were 25 ml/h. Bioluminescence intensity is represented by arbitrury unit.

The toxicity response model of the bioluminescence is derived from the Michaelis-Menten equation. The approach is based on the receptor theory, a concept used in toxicology to explain quantitative responses of living organisms to toxicants (26). In the model, the light quenching, q, represents the reaction velocity, v, and the K_m is dissociation constant,

$$q = q_{max} \cdot C_t/(K_s + C_t) \quad (7)$$

where q_{max} and C_t are defined as maximum quenching and toxicant concentration, respectively. As can be seen in Fig. 7 and 8, the immobilized cells were found to have lower q_{max} and higher K_s for both salicylate and 4-nitrophenol although numerical calculations were not made. The difference in kinetic parameters between free- and immobilized cells could be due to the transport system of chemicals. In all cases, the rate of quenching response to small quantities of salicylate and 4-nitrophenol represented as a slope of the curve at C_t=0 was shown to be increasing with increase in time.

Response Test with Immobilized Cells

Although the immobilized cells showed different kinetic parameters, the response of the bioluminescence to toxicants was evaluated with the immobilized luminescent bacteria for the application in real-time biosensing. The bioluminescence responses to 4-nitrophenol, salicylate and cadmium were measured and it was difficult to calibrate initial bioluminescence although luminescence was distributed as a dynamic quenching. For this reasons, the sensitivity was determined with the specific rate of bioluminescence quenching as described in eq.1.

Since Fig. 9 shows the typical bioluminescence quench-

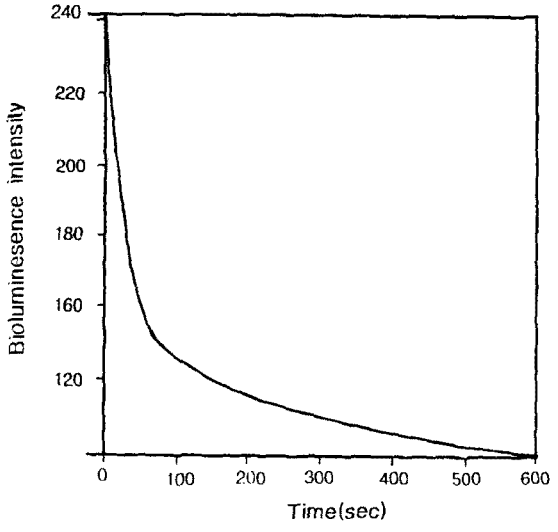

Fig. 9. Typical curve of bioluminescence quenching by 30 ppm of 4-nitrophenol.
Bioluminescence intensity is represented by arbitrury unit.

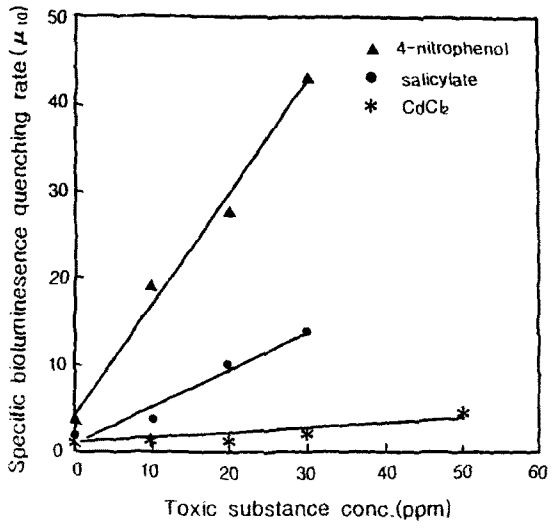

Fig. 10. Comparison of toxicity of 4-nitrophenol, salicylate and $CdCl_2$ as toxic substrates with immobilized cells.
Immobilized cells were exposed to above toxic substrates for 10 min and the specific bioluminescence quenching rates (μ_{10}) were calculated.

ing curve for 4-nitrophenol identifying a oppositely hyperbolic relationship between the quenching and time, the equation 1 was derived from

$$-dL/dt = \mu L \quad (8)$$

The important evaluation of determination by this equation is the sensitivity of the immobilized cells to the toxicants. The question is whether toxicants can be detected by the immobilized cells. The half of the bioluminescence is also calculated on the basis of a formula half-life=ln2/μ (data not shown). The resulting Fig. 10 shows the response sensitivity to typical toxicants used. The order of toxicity is similar to other work (9) although a quantitative measurements were not made. The range of a toxicant concentration applied to this work was selected for further biosensing development studies because of a good resolution.

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