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Continuous Water Toxicity Monitoring Using Immobilized *Photobacterium phosphoreum*

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Abstract Water toxicity monitoring based on the continuous cultivation of *Photobacterium phosphoreum* is presented. Normally, after 10 days of operation, a dark variant, which emits no light, appears and dominates the population, resulting in a rapid decrease in bioluminescence. Therefore, to overcome this problem, a fluidized-bed reactor is used in which alginate-immobilized cells are grown and leaking cells are continuously released into the effluent. Experimental results revealed that the dominance of dark variants was suppressed inside the immobilized beads, thereby mitigating the rapid loss of bioluminescence. Plus, a high dilution rate (1.2 h^{-1}) prevented the occurrence of other microbial contamination in the reactor. The concentration and bioluminescence of the released cells were sufficient to measure the water toxicity for more than 4 weeks.

Keywords: bioluminescence, dark variant, fluidized-bed reactor, immobilization, toxicity monitoring

INTRODUCTION

P. phosphoreum, a bioluminescent bacterium, produces a blue light, which can be easily observed with the naked eye in the dark. The mechanism of this light emission is an enzyme-mediated intracellular process as follows [1].

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + \text{light}$$

The process is very sensitive to physicochemical conditions due to the use of generated electrons in the metabolic processes [2]. As such, when placed in contact with toxic materials, such as phenol or heavy metals, the bacteria lose their bioluminescence very rapidly, *i.e.* within 5 min. Accordingly, this characteristic has been used to develop a toxicity monitoring system [3-8].

Bioluminescent bacteria have already been studied for monitoring water toxicity using an on-line mode [9]. The current authors also previously developed a water toxicity on-line monitoring system using the bioluminescent bacteria *P. phosphoreum* in a continuous stirred tank reactor (CSTR) [10]. During the operation of the CSTR, two problems were observed: that appearance of a mutant and the contamination of the reactor by other microbes. A dark variant with no light emission was observed after 10 days of cultivation, and the specific growth rate of the dark variant was faster than that of the wild type [10]. As a result, the population in the reactor shifted from normal bioluminescent bacteria to dark variants within only a few hours. In addition, frequent contamination of the reactor was also observed due to long-term continuous operation To solve these two problems, a method of entrapping bacteria is used with a high dilution rate during the reactor operation, thereby washing out any contaminant microorganisms growing outside the immobilized beads. The bioluminescent bacteria grow inside the immobilized beads and gradually leak out. Consequently, the growth of dark variants and their domination of the population inside the matrix are minimized, because the growth rates of both types of cell, *i.e.* bioluminescent as well as dark variants, inside the matrix are lower than those in the medium [11]. As a result, most of the cells in the effluent remain as bioluminescent cells, which can be used for continuous toxicity monitoring.

The current study has two objectives: first, to suppress the domination of dark variants and second, to monitor water toxicity using the cells released from an immobilized matrix during the operation of a fluidized-bed reactor.

MATERIALS AND METHODS

Strain and Culture Conditions

A frozen glycerol stock (20% v/v) of the *Photobacterium phosphoreum* KCTC 2852 strain was used as the

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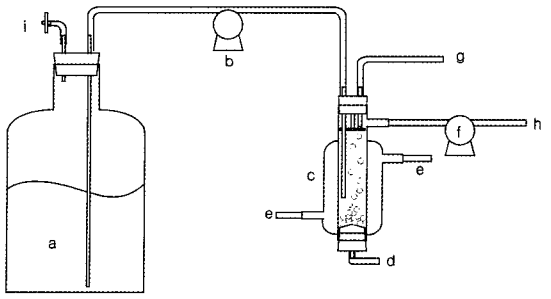


a: Culture medium b, f: Peristaltic pump c: Fluidized-bed reactor d: Air sparger
e: Wate jacket g: Air out h: Waste out i: Air filter

Fig. 1. Schematic diagram of fluidized-bed reactor for continuous culture.

inoculum. The culture medium consisted of 28.1 g/L NaCl, 1.6 g/L $CaCl_2 \cdot 2H_2O$, 4.8 g/L $MgCl_2 \cdot 6H_2O$, and 3 g/L yeast extract. The flask cultures were grown in a 250 mL Erlenmeyer flask at 18°C on a rotary shaker (Vision Scientific Co., Korea). The bioluminescence was measured using a luminometer (20e, Turner Designs, Sunnyvale, CA, USA). The unit of bioluminescence was defined as the relative light unit (RLU). The optical cell density was measured using a spectrometer UV-160A (Shimadzu Co., Japan).

Immobilization

The *P. phosphoreum* was cultivated in the culture medium and harvested by centrifugation (10,000 × g, 15 min, 4°C, T-124, Kontron, Germany). Next, the pellet was diluted to 10^4 cell/mL by resuspension with a 3% NaCl solution and mixed with sodium alginate (2.4%, final). This cell-alginate solution was then added to a 0.31 M $SrCl_2$ solution drop-by-drop using a peristaltic pump. Finally, the formed beads were allowed to harden for 30 min in the 0.31 M $SrCl_2$ solution [12].

Fluidized-bed Reactor Operation

The continuous culture of immobilized *P. phosphoreum* was performed in a fluidized-bed reactor with a 25 mL working volume. The reactor was operated at 18°C with 0.25 vvm of aeration and a dilution rate of 1.2 h^{-1} (Fig. 1). The bioluminescence of the released cells in the reactor effluent was measured using a luminometer, while the concentration of released cells was measured based on the optical density (OD_{600}).

Density and Ratio of Dark Variant in Beads

The number of dark-variant cells inside the beads was determined by dissolving the alginate matrix in 0.5 M hexasodium metaphosphate ($Na_6O_{18}P_6$) [9]. After serially diluting in a 2% NaCl solution, the mixture was then spread on an agar plate, incubated at 18°C for 36-40 h, and the colonies without bioluminescence counted.

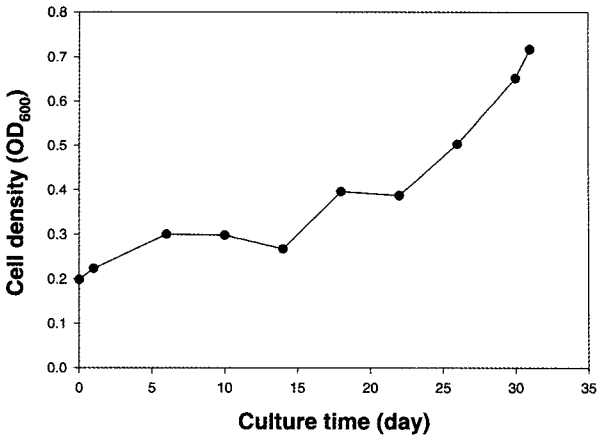


Fig. 2. Concentration of cells inside beads during continuous operation.

Toxicity Test

The toxicity test using the released cells was performed in a small reactor with a water jacket, as previously described [10]. $HgCl_2$ was used the model toxicant. The rate of decrease in the bioluminescence was then measured at various concentrations of $HgCl_2$.

RESULTS AND DISCUSSION

The initial concentration of cells in the alginate solution was kept low at 10^4 cell/mL to prevent excessive growth inside the beads. After immobilization, the number of cells inside the beads was about 100 cells per bead and the diameter of the beads ranged from 1.5~2.0 mm. The immobilized *P. phosphoreum* was then loaded into the fluidized-bed reactor and the medium fed continuously. After 24 h, microscopic observation revealed the formation of different sized microcolonies inside the beads (figure not shown), plus the microcolonies were located in the center of the beads, indicating no severe limitation of oxygen or nutrients.

During long-term operation, the growth of the cells inside the beads was slow (Fig. 2). For the first 15 days, the cell density remained relatively constant. The cell growth after 15 days was also very slow: only two-fold increase every 10 days. After 30 days of operation, no broken beads were observed under a microscope, also the beads were still hard. The slow cell growth during the 30 days of operation and hardness of the beads most likely prevented any rupturing of the beads. The cell density of the cells released from the immobilized beads was about 0.5 OD_{600} during the 35-day operation, except for day 3, when the bioluminescence suddenly increased suddenly in proportion to the cell density, indicating a higher rate of release for unknown reasons. However, the cell density in the effluent reached a steady state after 5 days, as the flow rate of the feeding medium was greater than the maximum growth rate of *P. phosphoreum*, hence the cells

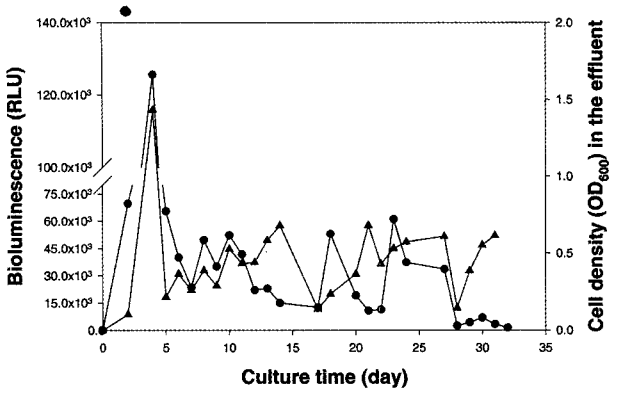


Fig. 3. Bioluminescence (●) and cells (▲) in effluent from fluidized-bed reactor.

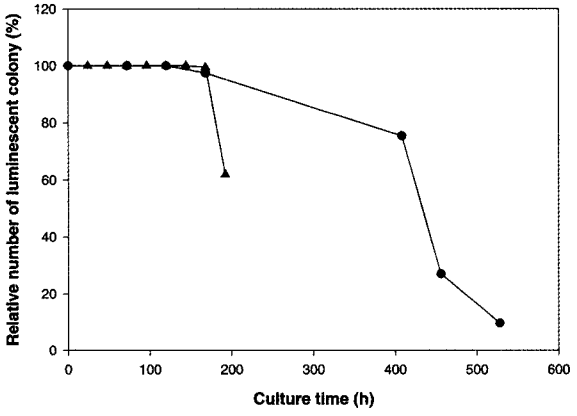
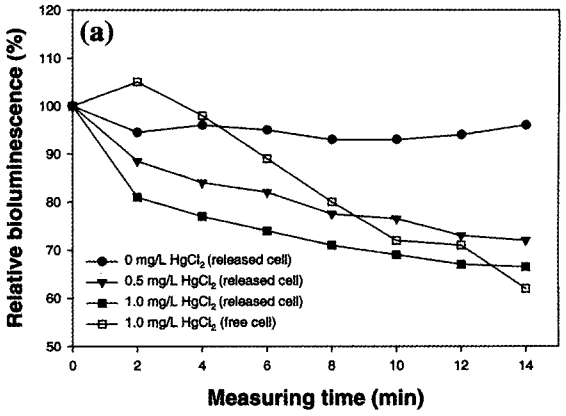


Fig. 4. Dark variant occurrence in fluidized bed reactor (●) and CSTR (▲). The CSTR was run at 0.1 h^{-1} using the same medium as in the fluidized-bed reactor.



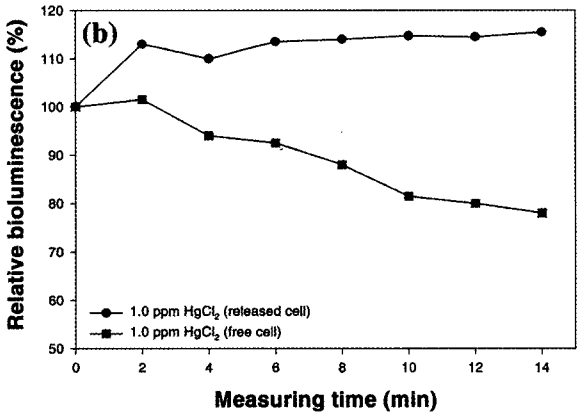


Fig. 5. Changes in bioluminescence of cells upon contact with $HgCl_2$. Cells were taken from the effluent on day 12 (a) and day 22 (b) of the continuous operation.

were continuously washed out. When the beads were examined by electron microscope, a few cells were observed attached to the surface of the beads. The intensity of the cell bioluminescence in the effluent was measured, and despite a fluctuation, the bioluminescence remained between 15-45 × 10^3 (RLU), which is sufficient to test the toxicity of the compounds.

During the continuous operation of the immobilized fluidized-bed reactor, the occurrence and domination of a "dark mutant" were suppressed (Fig. 4). For the initial 400 h of operation, more than 80% of the cell population was wild type, *i.e.*, light-emitting cells. In contrast, when cells were grown in a continuous stirred tank reactor (CSTR), a dark mutant, producing no light, dominated the population immediately after only 180 h of operation. In semi-continuous cultivation, dark mutant appeared at 7 day (0.6%) and dramatically increased 38.1% (8 day) and 100% (9 day), respectively. In addition, cell density did not decrease significantly during 9 days (13). There are two possible explanations for this observation. First, the mutation frequency may be higher in a CSTR than in an immobilized reactor. Second, the growth rate of the cells in an immobilized matrix is lower than that in a CSTR, therefore, it takes more time for a 'dark mutant' to dominate the population in an immobilized matrix. At this point, it is difficult to determine the mutation frequency in a CSTR or immobilized reactor. Thus, assuming that the mutation frequency is the same in both reactors, the second explanation would seem to explain the delay in mutant domination. Similar observations have also been reported by other researchers [11,14].

The released cells were used to determine the toxicity of mercury in the water (Fig. 5). The bioluminescence of the released cells in the effluent of the reactor was measured in a separate chamber after adding $HgCl_2$. The bioluminescence of the cells in the chamber remained constant during the measuring time (14 min). When 0.5 mg/L of $HgCl_2$ was added, there was a noticeable decrease in the bioluminescence. In addition, a sharp decrease was also observed after 2 min of contact with 1 mg/L $HgCl_2$.

Free cells, grown in a flask in the same medium, exhibited a sharper decrease in bioluminescence. The released cells were more sensitive to $HgCl_2$. Because free cells are active, which are impervious to toxic materials [15]. However, no significant difference was observed between

the responses of the free cells and the released cells. After 22 days of operation, the released cells also exhibited a similar response to 1 mg/L $HgCl_2$ (Fig. 5(b)), confirming that the cells released from the immobilized bioreactor could still be used to monitor the toxicity of the compounds in the water.

Accordingly, the current paper outlined the development of a fluidized immobilized reactor for the continuous on-line monitoring of toxic compounds in water. The results showed that rapid domination by a dark mutant, non light-emitting mutant, could be suppressed, enabling bioluminescence to be measured for more than 30 days. Furthermore, operating at a higher dilution rate also facilitated long-term operation by minimizing the growth of other contaminating microbes in the effluent.

CONCLUSION

The main problems of continuous toxicity monitoring based on the continuous cultivation of bioluminescent bacteria are related to the contamination of the reactor by other microbes and the occurrence of a dark variant, non light-emitting bacteria. Therefore, to overcome this problem, a fluidized-bed reactor was used where the cells were immobilized, cultured, and continuously released into the medium. Domination by a cell population of dark variants was suppressed until 20 days of continuous operation. The cells released from the immobilized matrix into the effluent exhibited sufficient bioluminescence to detect as little as 0.5 mg/L of the model toxic compound $HgCl_2$. Accordingly, the current results demonstrate potential of using a fluidized-bed reactor with immobilized bioluminescent bacteria for long-term toxicity monitoring in the field.

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