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A NEWLY DEVELOPED CONTINUOUS TOXICITY TEST SYSTEM USING A LUMINOUSLY MODIFIED TERRESTRIAL BACTERIUM

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Summary

Freshwater borne bacteria transformed with *luxAB*-containing plasmid were optimized for the toxicity tests of various organic carbons and heavy metals. The EC_{50} values obtained from tests using the most sensitive bacterium to toxicants, YH9-RC, revealed to be much less than those from the Microtox®. In addition, some physiological characteristics of this bacterium under the toxic stress conditions such as potential bioluminescence, specific growth rate, and intracellular ATP contents, reproducibly and reliably correlated to the toxicity of the chemicals exposed. The higher concentrations of COD in wastewater samples, the lower EC_{50} values, therefore the developed toxicity test was found to be easily applicable to the toxicity test for wastewater samples and effluents. The conditions for constructing 384-multiwell plate containing freeze-dried bacterium were also optimized through the addition of 0.16 M trehalose before freeze-drying. Consequently, the advanced test system featuring a continuous measurement of the toxicity, an automated real-time monitoring of its results, and an alerting function was designed and constructed in combination with the microbiological, mechanical, and electronic compartment.

Introduction

The impacts of industrial effluents and sewage on aquatic ecosystems such as rivers, lakes, and groundwaters have become more serious with the development of various industries in Korea. Considering the conservation of environment and the protection of human health, effluent standards are determined for routine environmental parameters such as BOD and COD, and for 24 chemicals including heavy metals and organic solvents. However, because surface water and wastewater consist of complex mixtures containing many different chemicals and most of which have yet to be chemically and toxicologically characterized, the conventional chemical-screen method is of limited value in the context of toxicity. Therefore, the USA and several EU countries have evaluated total toxicity that may include unknown effects of new chemicals (4, 13, 14).

Several bioassays have been developed to determine the toxicity of materials that produce harmful effects on the biological system, using fish, protozoa, algae, and other freshwater and marine organisms (7, 9, 12). However, most of these tests are composed of relatively long-term observation processes and expensive, and they often require the time-consuming propagation of test organisms, while the rate at which anthropogenic chemicals are developed and distributed is not matched by the rate of increased scientific or public awareness. In contrast to animal or algae-based toxicity measurement systems, bacterial systems are particularly applicable to rapid toxicity testing on account of their ease of use, low cost, and the statistical advantage in using a large number of bacteria instead of the small number of organisms associated with other bioassays (3).

In this respect, the recently developed Microtox® system using marine bioluminescent bacterium *Vibrio fischeri* has been suggested to be a useful tool. However, it was revealed a poor indicator of toxicity for effluents containing certain compounds and a lower degree of sensitivity compared to a toxicity test using aquatic non-invertebrate (2). In addition, it has been reported that the adjustment of

test samples to 2% NaCl for the Microtox® assay alter the mode of action or binding of the some chemicals such as 2,4-dichlorophenol to cells, thus may not precisely measure the toxicity of chemicals in terrestrial environments (11). Therefore, a toxicity test similar to Microtox® but based on aquatic bacteria rather than marine *V. fischeri* would be more relevant for applying aquatic toxicity test. This can be obtained by insertion of the *lux* genes encoding luciferase into the aquatic bacterium.

Lux based bioassays can be used as a biological early warning system (BEWS) to monitor water quality. However, as the measurement of toxicity has been routinely carried out by discontinuous manner (batch operation) in the Microtox® bioassay system, it is impossible to apply the current Microtox® system to the field of BEWS. The reason is that continuity and automation are prerequisite for the purpose of BEWS.

The main purpose of this study is to develop an advanced continuous toxicity test system by using *lux*-marked terrestrial bacterium. In this study, we focused on development of the advanced toxicity test system by constructing 384-multiwell plate containing freeze-dried luminescent bacterium, instrument for continuous measurement of bioluminescence, and the computer program (BactoTox®) capable of real-time monitoring of toxicity and telecommunication.

Materials and methods

Construction of bioluminescent strain

A freshwater bacterium Y4R-C, which was sensitive to various volatile organic carbons (VOC's) and heavy metals, was isolated from Kyoungan stream and subsequently mutagenized to be highly sensitive (designated as UV2) (8). Using the same method, strain YH9-RC, which was higher sensitive to VOC's and heavy metals than UV2 was screened from groundwater used for natural mineral water. Bioluminescent strains were obtained by mating *E. coli* S17-1 (λpir) pUT*luxAB* (Tc^R) with the recipient cells (Rif^R) using the filter conjugation method (6).

Toxicity test

Toxicity tests were performed with the 4 luminescent bacterium (*V. fischeri*, Y4R-C, UV2, and YH9-RC) as the test microorganisms to observe the reduction of their bioluminescence emission instead of the observation of their death when they contact with the toxicants. *V. fischeri* was used for comparing the developed toxicity test with the Microtox® assay. Toxicity tests were designed for VOC's, heavy metals, wastewater, and effluents. Each test consisted of one control and series of different concentrations of the chemicals and serial dilutions of wastewater samples. Bioluminescence was measured in each test with a luminometer (TD-20/20, Turner designs, CA, USA). The results of the test were expressed as EC_{50}, the effective concentration or dilution ratio of a sample that causes a 50% reduction of the luminescence emitted by a test bacterium. The 5-, 15- (or 10-), and 30-min EC_{50} were calculated for each test using a linear regression analysis. During toxicity test, besides measuring EC_{50} by reduction of luminescence, EC_{50} (ATP) and EC_{50} (μ) was measured by measuring ATP content in the cells and specific growth rate, respectively. ATP content in cells was determined by ATP detection kits consisting of luciferin-luciferase and a luminometer.

Construction of continuous toxicity test system

A brief description of the newly developed automated and continuous toxicity test system is given here, full details, including drawings of robot arms for stage transport, electronic circuit diagrams, pumping apparatus, computer program, and user guide manual can be obtained from the corresponding author. A schematic diagram of the system is shown in Fig. 1. For measuring toxicity automatically, the 384-multiwell plate as a cartridge for supplying luminescent bacterium was constructed with exponential culture of luminescent cells by freeze-drying them and addition of trehalose known as protective agents for cell. The system was built with the combination of sample

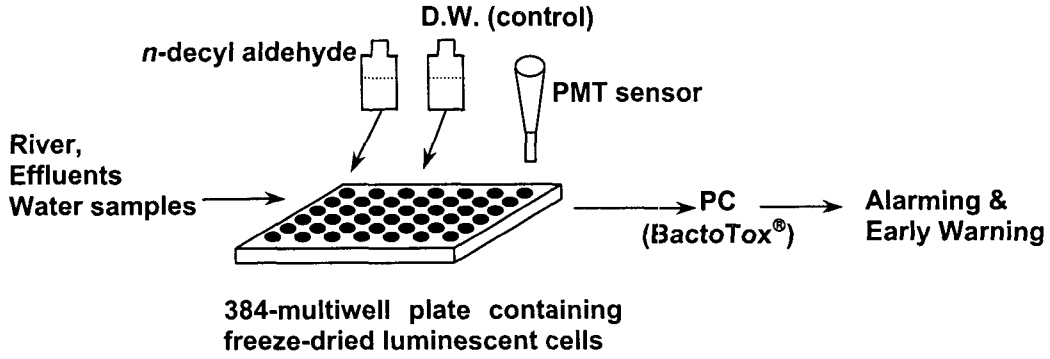

Fig. 1. Schematic diagram of continuous toxicity test system

supplier, solution dispenser compartment, 384-multiwell cartridge storage tray, cartridge loading compartment, photomultiplier tube (PMT) sensor, PMT sensor transporting compartment, temperature controller, multi-controller compartment including RS232C serial port, and computer (Pentium II, IBM PC) running the developed BactoTox® software. The BactoTox® software which has novel features for on-line monitoring of toxicity and alerting function was developed by using the Visual Basic program on the basis of 32-bit operating system.

Results and Discussion

Superiority of YH9-RC as a test bacterium for toxicity measurement

The results of the toxicity testing were expressed EC_{50} values which represent the amount of phenol and various heavy metals required to reduce light output by 50% (Table 1).

Table 1. Toxicity of various chemicals by using *V. fischeri* and luminously modified aquatic bacteria.

	EC_{50} (5 min, mg/l)				EC_{50} (15 min, mg/l)				EC_{50} (30 min, mg/l)			
	V.fischeri	Y4R-C	UV2	**YH9-RC**	*V.fischeri*	Y4R-C	UV2	**YH9-RC**	*V.fischeri*	Y4R-C	UV2	**YH9-RC**
Phenol	115.6	88.0	75.0	**26.5**	105.1	76.2	71.5	**12.0**	63.1	71.6	69.0	**12.7**
Al	46.1	6.0	8.7	**0.93**	45.8	2.9	4.1	**Nd**	41.5	1.9	3.3	**nd**
As	65.6	30.6	14.1	**28.4**	74.1	36.1	18.2	**9.3**	64.4	25.9	15.5	**4.3**
Cd	71.4	49.8	21.2	**4.62**	56.1	40.2	19.0	**1.81**	41.4	24.3	17.5	**1.10**
Co	71.6	2.7	12.7	**1.11**	54.4	2.4	5.5	**1.84**	41.4	2.6	3.6	**1.96**
Cr(VI)	10.3	7.6	23.9	**5.88**	10.4	7.6	29.7	**7.53**	8.8	8.4	29.5	**6.29**
Fe	55.1	15.3	45.9	**6.69**	54.3	9.9	43.5	**9.22**	51.3	5.4	37.0	**11.0**
Hg	3.5	2.9	2.5	**0.31**	3.1	2.6	1.9	**0.22**	3.1	2.4	1.4	**0.19**
Mn	336.6	98.7	144.1	**25.0**	747.1	95.5	161.6	**nd**	300.8	95.0	178.6	**nd**
Se	90.3	86.2	102.3	**29.5**	81.6	58.8	88.6	**nd**	70.5	70.1	122.5	**52.6**
Zn	41.6	27.3	16.8	**1.30**	39.8	20.6	16.1	**1.34**	38.8	14.4	14.7	**1.29**

The constructed luminescent bacteria, Y4R-C, UV2 and YH9-RC were generally more sensitive to various toxic chemicals than marine *V. fischeri* used for Microtox® assay. Especially, comparison of the toxicity assay based on *luxAB*-marked YH9-RC to Microtox®, showed that EC_{50} values calculated for 11 chemicals were significantly lower than Microtox® assay ($p < 0.001$). Therefore, the use of YH9-RC strain in acute toxicity assessment is a potential useful tool in surface water toxicity monitoring.

The decrease in light output in the given toxicity test might be due to the reduction of luciferase activity itself or the decrease of whole cell metabolic activity. Therefore, during the toxicity test for *p*-xylene and mercury, we measured the luciferase activity, the specific growth rate (μ), and ATP contents of the cells. There was no significant change in luciferase activity in cells during toxicity test, while decrease in luminescence in test strains due to the presence of toxic chemicals has been found to be correlated with the degree of decrease of cellular ATP contents and specific growth rate (data not shown). Cellular ATP content has been shown to be a good estimate of microbial biomass, and reduction in ATP content can be considered as a criterion for microbial response to toxicants (15). The decrease in light output suggested that the metabolism of the cell was being disrupted by toxicants, therefore, the measured EC_{50} (luminescence) could reflect the whole cell toxicity.

Toxicity test in wastewater samples

In order to define whether the developed acute toxicity system using YH9-RC as a test bacterim can be applied to measurement of wastewater toxicity, the toxicity test of the raw wastewater, aerobic treatment tank, and effluent samples in a food industry plant was carried out and resulting EC50 values (as percent values) are represented in Table 2. One hundred percent wastewater sample is undiluted sample. Therefore, the higher EC_{50} values, the less acutely toxic the water samples. The EC_{50} values in untreated raw wastewater sample were lowest, while those in effluent were highest. The higher concentrations of COD in wastewater samples, the lower EC_{50} values. From this point, the developed toxicity test was found to be easily applicable to the filed of toxicity test for wastewater samples and effluents. This study has shown that *lux* marked bacterium YH9-RC can be used a bioassay to assess the ecotoxicity of wastewater and effluent samples predominantly contaminated with COD compound. Asami *et al* (1996) (1) suggested the following three categories based on EC_{50} values: high toxicity for cases where EC_{50} is not more than 20%, low toxicity for cases where EC_{50} is more than 20% and not more than 100%, and little or no toxicity in which EC_{50} is more than 100%. From these categories, it was shown that all tested samples were found to have low toxicity.

Table 2. Toxicity of wastewater samples by using YH9-RC as a test bacterium.

Samples	EC50 (%)		
	5 min	10 min	30 min
Raw wastewater	23.9 ± 12.8	23.3 ± 12.3	38.5 ± 18.1
Aeration tank	45.5 ± 9.8	31.8 ± 7.9	39.3 ± 12.3
Effluents	76.7 ± 14.9	57.9 ± 10.7	50.5 ± 10.8

Freeze-drying conditions and construction of 384-multiwell plate

The developed advanced toxicity test system uses 384-multiwell plate containing freeze-dried luminescent cells as a continuous supplier for test bacterium. The schematic diagram of procedures for

constructing 384 multi-well plate and freeze-drying conditions are shown in Fig 2. Freeze-drying has been generally used for long-term preservation of microorganisms. However, we have found that freeze-drying of luminescent bacteria brings out decreased luminescence and viability of them. Some authors have shown that freeze-drying in the presence of trehalose or sucrose increase the survival of bacterial samples (5, 10). Addition of 0.16 M trehalose before freeze-drying was very effective to minimize the adverse effect of freeze-drying. When the bacterium was freeze-dried with 0.16 M trehalose, 48.7% of bioluminescence and 54.1% of culturability were restored. Even though all cells were not fully recovered by addition of trehalose, luminescence emitted by freeze-dried cells were sufficient for continuous toxicity test, thus the 384-multiwell plate could apply to the continuous toxicity test system.

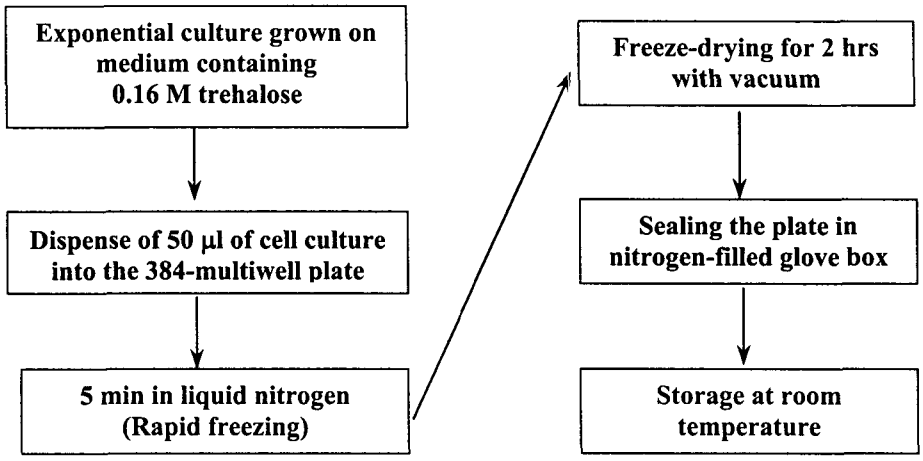

Fig. 2. Procedures for constructing 384-multiwell plate and freeze-drying conditions

Characteristics of the advanced continuous toxicity test system

The main characteristic of the developed toxicity test system is to monitor toxicity of samples automatically and continuously without operator. The system includes twelve 384-multiwell plates, which can be maintained for a month without operator if toxicity test is performed at a 10-min interval When a water sample is pumped from river or stream, solution dispenser compartment start to dispense a water sample and *n*-decyl aldehyde into a well containing luminescent bacterium. After incubating for 10 min at 25℃, the PMT sensor start to measure luminescence emitted from each well, thereafter, ADC compartment convert the analog values of PMT sensor to the digital signals and RS232C serial port transmit the digital values to a PC loading BactoTox® program. The main functions of Bactotox® software are to transform of bioluminescence values into the defined data structure for on-line monitoring, to give the alarm in case of high toxicity, and to communicate with main server via modem or internet. A newly developed continuous toxicity test system integrated based on these features can be used for forecasting water pollution by a sudden input of toxic materials into water environment.

In conclusion, this work demonstrates that developed simple, cost-effective, automatic, and continuous toxicity test system are particularly suited for biomonitoring of domestic or industrial wastewaters as well as recipient waters.

Acknowledgement

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