Analyses of Factors to Affect the Bioassay System Using Luminescent Bacterium *Vibrio fischeri*

Shinichi Nagata* and Xiaojian Zhou

*Environmental Biochemistry Group, Research Center for Inland Seas, Kobe University, 5–1–1 Fukaeminami, Higashinada, Kobe 658–0022, Japan*

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Analytical conditions for evaluating the chemical toxicity were examined using luminescent bacterium *Vibrio fischeri* (V. fischeri) deutsche sammlung von microorganismen (DSM) 7151, taking the medium composition into consideration. The manipulation of the cells for optimal assay was obtained as follows; the cells were grown in luminescence (LM) medium containing 3 g/l of glycerol for 18 hr. They were washed twice with artificial seawater (ASW) and then diluted about 128 folds by ASW, corresponding to 1.0–1.5 × 10⁶ cfu/ml. Using 4,5-dichloro-2-(n-octyl)-4-isothiazolin-3-one (SeaNine 211) as a representative toxic chemical, we evaluated the extent of its toxicity in terms of present method, where 50% effective concentration (EC₅₀) resulted in 0.51 and 0.35 mg/l at 15 and 30 min of incubations, respectively. Sensitivity of present assay was higher than those of traditional ones by over 2 folds from the comparison of EC₅₀ for Cu²⁺. The results show that the present assay using freshly cultured *V. fischeri* has not only high sensitivity and reproducibility but also simplicity to estimate the contamination by chemicals.

**Key words** —— *Vibrio fischeri* DSM 7151, bioluminescence, bioassay, 4,5-dichloro-2-(n-octyl)-4-isothiazolin-3-one

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*To whom correspondence should be addressed: Environmental Biochemistry Group, Research Center for Inland Seas, Kobe University, 5–1–1 Fukaeminami, Higashinada, Kobe 658–0022, Japan. Tel. & Fax: +81-78-431-6342; E-mail: nagata@maritime.kobe-u.ac.jp
Furthermore, BLI of test kits’ reagents tends to be decreased accompanied with the increase of storage time.

Owing to these reasons, some studies have been made to achieve a greater flexibility in the selection of an appropriate test system for the assessments of environmental toxicity. Thus, the substitutable and flexible methods under similar conditions as natural environment have been expected to provide more accurate evaluation for pollutants. According to previous studies, manipulation of cells have been discussed whether medium components should be eliminated to avoid its interference of cellular activity although Lajoie et al. claimed no requirement of cell washing. Thus, it seems to be of value to address the confirmation of assay system through fundamental analyses of several kinds of conditions.

In the present study, therefore, we tried to clarify the suitable conditions of the bioluminescent assay using V. fischeri with the aim of practical applicability to detect the toxicity of chemicals sensitively. Particularly, we focused on the influence of NaCl concentrations against the sensitivity of present method, since environmental samples possess wide concentration ranges of NaCl and V. fischeri is marine bacterium to require NaCl for its optimal activity.

**MATERIALS AND METHODS**

**Culture Conditions** —— A strain used in this study was V. fischeri deutsche sammluing von microorganismen (DSM) 7151, which was grown in two kinds of media. One was a LB medium which contains 0.5% yeast extract (Difco Laboratories, Detroit, MI, U.S.A.), 1.0% tryptone (Difco Laboratories), and 0.5 M NaCl. The other was luminescence (LM) medium, in which 0.5% yeast extract (Difco Laboratories), 0.5% tryptone (Difco Laboratories), 0.1% CaCO₃, and 0.3% glycerol were involved in artificial seawater (ASW, JIS K-2510) in place of actual seawater used in original LM medium. pH of both media was adjusted to 7.0 by NaOH. The cells were incubated in media with a rotary shaker (120 rpm) at 30°C by adding 1%(v/v) of pre-cultures with 13 and 24 hr of incubations in LB and LM media, respectively. Growth was determined by measuring optical density at 650 nm (OD₆₅₀) using a spectrophotometer (DU 640, Beckman, Fullerton, CA, U.S.A.).

**Bioluminescence Measurements** —— The cells grown in both media were taken up at an appropriate time and subsequently separated by centrifugation (10000 × g, 1 min, 4°C), followed by washing with 0.5 M NaCl or ASW. BLI was measured for 0.1 min integral time by luminescence reader (BLR-201, Aloka, Tokyo, Japan). Cell suspensions were prepared as either 0.2 or 0.5 ml volume in liquid scintillation vial (Wheaton, Millville, NJ, U.S.A.), each of which corresponds to higher and lower cell densities, respectively.

As toxic chemicals, we used 4,5-dichloro-2-(n-octyl)-4-isothiazolin-3-one (SeaNine 211), CuSO₄, tributyltin chloride (TBT-Cl), 3-iodo-4-tert-butylamino-6-cyclopropylamino-s-triazine (Irgarol 1051). The efficacy and mechanism of them are as follows; Toxic effect of Cu²⁺ is suppression of mitosis through glutathione reduction and destroy of cellular defense against oxygen-free radicals. SeaNine 211 is typical inhibitor for settlements of bacteria and algae. TBT is well known as one of the most significant hazardous compounds to aquatic organisms through neurotoxic, hepatotoxic, immunosuppressive and hormone disruptive activities. In addition, Irgarol and IPBC are inhibitors for photosystem-II and for acetylcholinesterase on animals, respectively.

Each chemical dissolved in dimethyl sulfoxide (DMSO) were added to the cell suspension at final concentration of 1%. At the same time, we prepared control without chemicals, for both of which the changes of BLI were followed.

**Calculations** —— To determine the toxicity of chemicals to V. fischeri, percentage of inhibition efficiency [INH (%)] was used in this study. The values of INH (%) were calculated from BLI changes between samples and controls as follows;

\[
\text{INH(%) } = \left[1 - \frac{\text{IT}_t \times \text{IC}_0}{\text{IT}_0 \times \text{IC}_t}\right] \times 100
\]

where INH (%): Percentage of INH.

\(\text{IT}_t\): BLI of the sample after the contact time (t) with chemicals.

\(\text{IT}_0\): Initial BLI of the sample.

\(\text{IC}_t\): BLI of the control after incubation time (t).

\(\text{IC}_0\): Initial BLI of the control.

The data of INH (%) in this text are the averages of at least two independent measurements. The value of 50% effective concentration (EC₅₀) was obtained as the concentration corresponding to INH (%) = 50 in the relationship between INH (%) and concentration of chemicals.
Growth Condition of \textit{V. fischeri} for High Intensity of Bioluminescence

When the cells were grown in a LB medium, they reached the stationary phase of growth after 14–18 hr of incubation. Bioluminescence emission from the cells was observed accompanied with cell growth, as shown in Fig. 1A. Incubation time of 11–12 hr afforded the maximum of BLI and longer incubation time, > 12 hr, led to rapid decrease. Similar tendency was observed in a LM medium, in which BLI increased with the incubation time and attained at maximum at 18–22 hr of incubation, as shown in Fig. 1B. The incubation time to reach the maximum of BLI in LM medium was different from that of LB medium. In addition, BLI from the cells grown in LM medium resulted in four-fold higher than that in LB medium.

The difference of composition between two media is mainly regarded as the inclusion of glycerol and CaCO$_3$ in LM medium. In addition, ASW was used in LM medium in place of 0.5 M NaCl in LB medium. About these different factors, first we tried to examine the effect of glycerol in growth medium to clarify the stimulative effect on BLI of the present strain DSM 7151. As shown in Fig. 2, the cells grown in both LB and LM media had enhanced BLI with the increase of glycerol concentra-

\textbf{RESULTS AND DISCUSSION}

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tion in medium. The addition of glycerol led to over 2 folds higher BLI compared to those without addition of glycerol for both media, in which the maximum was observed in the presence of 3 g/l of glycerol. To make clear the difference of both media, we analyzed in detail BLI of the cells grown in LB medium, in which bioluminescence maximum of cells grown with glycerol was enhanced by 2.4 folds compared to that without glycerol, as shown in Table 1.

Next, the effects of CaCO₃ addition as well as the usage of ASW in place of 0.5 M NaCl were examined. With an increase of CaCO₃ concentration in LB medium BLI of the cells increased until 1 g/l, but it became constant over 1 g/l of CaCO₃ (data not shown). The addition of 1 g/l of CaCO₃ brought about 1.5 folds enhancement of BLI as shown in Table 1. Furthermore, BLI increased from 2000 to 2800 kcpm/ml min, if ASW was used in place of 0.5 M NaCl in LB medium (Table 1). Thus it became evident that the addition of glycerol and CaCO₃ as well as the usage of ASW led to significant increase of BLI of the strain DSM 7151. It seems to be of value to note that the use of different concentration of tryptone in both media, 5 and 10 g/l in LM and LB media, respectively, was negligible, since little difference of BLI was observed in the cells grown in LM medium and LB medium with 0.3% glycerol and 0.1% CaCO₃ in ASW as shown in Fig. 1B.

To use the present bioassay efficiently, BLI of V. fischeri DSM 7151 should be stable and relatively high to get reliable data, which leads to high sensitivity in the real toxicity accompanied with improved signal to noise ratios. The utilization of LM medium for growth brought about feasible conditions to obtain high BLI for the present strain DSM 7151. Taking the steep decrease of BLI after 22 hr of incubation into consideration, cells grown in LM medium for 18 hr are recommended to be used for the achievement of the most suitable and sensitive assay.

### Table 1. Effect of Medium Composition on BLI of V. fischeri

<table>
<thead>
<tr>
<th>Medium</th>
<th>Maximal BLI (kcpm/ml min)</th>
<th>Enhancement folds</th>
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<tbody>
<tr>
<td>LB</td>
<td>600 (11)</td>
<td>1.00</td>
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<tr>
<td>LB + Glycerol</td>
<td>1428 (15)</td>
<td>2.38</td>
</tr>
<tr>
<td>LB + Glycerol + CaCO₃</td>
<td>2007 (16)</td>
<td>3.35</td>
</tr>
<tr>
<td>LB + Glycerol + CaCO₃ + ASW</td>
<td>2777 (20)</td>
<td>4.63</td>
</tr>
<tr>
<td>LM</td>
<td>2657 (20)</td>
<td>4.43</td>
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</tbody>
</table>

a) Values in parentheses indicate the incubation time to reach maximal BLI (hr).

### Interference Effect of Medium Composition

An attempt was made to determine how the cells with active luminescent ability should be manipulated. Ren et al. showed that the color of growth medium affected some sensitivity of luminescent bacteria unless medium compositions were eliminated before use. In this connection, we examined the influence of medium components by using the cells diluted by 100 folds with growth medium freshly prepared as well as with ASW. For sensitivity comparison, we used 0.3 mg/l of SeaNine 211 which is lower than the following concentration of 0.4 mg/l. As shown in Fig. 3, INH (%) in the cells diluted by fresh medium and ASW resulted in 16 and 23% at 30 min of incubation, respectively (Fig. 3, a and b). That is to say, systems possessing high portions of medium components showed low sensitivity. In addition, it appears to be of interest to point out that inhibition percentages were constant or rather reduced with an increase of the incubation time, suggesting that there might be some problems to affect the sensitivity if medium components are included.

To determine the interference effect of medium components on the sensitivity of present bioassay system, we tried to eliminate them more perfectly through washing processes. The cultured cells after centrifugation were suspended in ASW, which still contained medium components (Fig. 3, c). After 30 min of incubation we observed the enhancement of sensitivity through centrifugation and the use of ASW, from 23 to 30%, as realized from the comparison of b and c in Fig. 3. By increasing the number of cell washing, the sensitivity increased and the optimum was observed after two times washing. Furthermore, the inhibition percentages increased with the increase of incubation time for the cells washed one or two times, which indicates that such state of cells reflects accurately toxicity of chemicals. Over three times washing, however, led to the decrease of sensitivity, probably due to significant cell damage.
of the strain DSM 7151.

In this connection, we also performed cell washing with ASW or 0.5 M NaCl solution to make clear the influence of washing solution. As shown in Table 2, the use of ASW led to minor reduction of BLI compared to that of 0.5 M NaCl solution, irrespective of whether the cells were grown in LM or LB medium. From the comparison of growth medium, more serious reduction of BLI was observed in the cells grown in LB medium in comparison with that of LM medium (Table 2). In conclusion, the cells that were grown in LM medium and washed two times with ASW resulted in the highest sensitivity among them, probably due to not only low content of medium components but also little damage of cells through centrifugation with ASW.

**Effect of Cell Dilution**

The viable cell numbers of *V. fischeri* grown in LM medium for 18 hr were around $1.5 \times 10^8$ cfu/ml. To make the cell assay more sensitively, we tried to obtain a suitable cell density through dilution of cells using ASW. INH (%) of BLI was determined at 15 and 30 min of incubations in the presence of 0.4 mg/l SeaNin 211. As shown in Fig. 4, the sensitivity of present assay was increased almost linearly with the increase of dilution folds by ASW. Over 128 folds dilution, INH (%) decreased gradually, in which the significant deviation of the data was also observed. As a consequence, dilution of the cells by 128 folds with ASW afforded the highest sensitivity. After about 128 folds dilution of the cells by ASW, the cell density and BLI resulted in about $1.0 - 1.5 \times 10^6$ cfu/ml and 8–12 kcpm/ml•min, respectively.

**Effect of NaCl Concentration in the Assay Medium**

After washing procedure, BLI of the cells suspended in various concentration of NaCl was followed at 0.2, 15, 30 and 60 min of incubations. At each incubation time, BLI of cells increased with an increase of NaCl concentration until 0.3–0.4 M and decreased over 0.5 M as shown in Fig. 5. In the presence of 0.1 M NaCl, cellular BLI was not detectable and at the same time it was too low to apply the present bioassay system at > 1 M NaCl. Relatively stable and high BLI of the strain DSM 7151 was observed in 0.3–0.5 M NaCl in the incubation time of 0.2–60 min, which suggests that the present system seems to be enough sensitive to apply for samples containing toxic chemicals.

**Table 2. Effect of Cell Washing with ASW or 0.5 M NaCl on the BLI**

<table>
<thead>
<tr>
<th></th>
<th>LB medium$^a$</th>
<th>LM medium$^b$</th>
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<tbody>
<tr>
<td>Before washing</td>
<td>410 ± 28</td>
<td>2500 ± 113</td>
</tr>
<tr>
<td>After washing with ASW</td>
<td>134 ± 12</td>
<td>1827 ± 84</td>
</tr>
<tr>
<td>After washing with 0.5 M NaCl</td>
<td>87 ± 9</td>
<td>1333 ± 22</td>
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</table>

$^a$ Washing was performed twice. Values of BLI (kcpm/ml•min) are the average ± standard deviations from three independent experiments. $^b$ Incubation time for growth was 8 hr. $^c$ Incubation time for growth was 18 hr.
As a comparison, BLI of the cells suspended in ASW was also followed and shown in Fig. 5. At each incubation time of 0.2, 15, 30 and 60 min, BLI of the cells suspended in ASW was higher than those in the presence of NaCl (Fig. 5). The same was true for the stability of BLI of the present strain. Furthermore, the metabolic activity of cells led to the increase of pH in NaCl solution, namely from 7.2 to 7.8 during 2 hr of incubation, while pH changes of cell suspension in ASW was negligible, probably due to its buffering effect. Present finding might suggest that more comfortable hydrogen-ionic and osmotic environment for cells was created in ASW compared to NaCl solution.13,23) Thus, it might be concluded that the usage of ASW has an obvious advantage for maintenance of high BLI in comparison with NaCl solution.

Fig. 4. Effect of Cell Dilution on the Sensitivity against SeaNine 211

Cells grown in LM medium for 18 hr were washed twice and diluted 8–512 folds with ASW. Final concentration of SeaNine 211 added to each cell suspension was 0.4 mg/l. INH (%) at 15 (closed circles) and 30 min (open circles) of incubations was described by the averages with standard deviations from three independent experiments.

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**EC₅₀ of Some Chemicals Based on the Present Assay System**

An attempt was made to examine whether the present assay system is applicable for actual contamination by toxic chemicals. After mixing different concentrations of SeaNine 211 into cell suspensions of *V. fischeri* thus manipulated, changes of BLI were analyzed as shown in Fig. 6, where BLI was
We compared EC50 values of Cu2+ with chemicals. alteration was observed depending on the kinds of respectively. In the present assay, however, such alteration was observed depending on the kinds of TBT-Cl and IPBC belong to second or third groups, CuSO4, 0.51 and 0.71 mg/l of EC50 values at previous report that TBT-Cl is extremely toxic.6,12,23) remarkable increase, although TBT-Cl showed quite tent. In contrast, TBT-Cl and IPBC did not afford so indicating that toxicity of this chemical tends to be persistent. In contrast, TBT-Cl and IPBC did not afford so remarkable increase, although TBT-Cl showed quite high toxicity, which is in good accordance with the previous report that TBT-Cl is extremely toxic.6,12,23) In response to the changes of EC50 for different chemicals, there exists, in general, three types of Light Level-Time Response Curves (LLRCs), those of which are classified from the alteration of BLI such as sharp drops and then constant, decrease almost linearly, and their intermediate. As the representative example, respective ones are phenols, heavy metals, and organic chemicals in order. Based on this classification, SeaNine 211 and CuSO4 or TBT-Cl and IPBC belong to second or third groups, respectively. In the present assay, however, such alteration was observed depending on the kinds of chemicals.

To evaluate the superiority of present assay in sensitivity, we compared EC50 values of Cu2+ with those obtained by other methods, in which EC50 with > 10 mg/l were observed through the inhibitory analyses of nitrification, respirometry, and enzyme activities.11) These reasonable and sensitive data described here suggest that present method is quantitatively applicable for detection of such toxic chemicals.

Acknowledgements We would like to appreciate for Dr. H. Okamura, Kobe University, for providing chemicals and valuable suggestions.

Table 3. EC50 of Some Typical Chemicals Obtained from Present Assay System (mg/l)6

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>SeaNine 211</th>
<th>CuSO4</th>
<th>TBT-Cl</th>
<th>IPBC</th>
<th>Irgarol 1051</th>
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<tr>
<td>15 min</td>
<td>0.51 ± 0.022</td>
<td>0.77 ± 0.06</td>
<td>0.018 ± 0.0013</td>
<td>9.83 ± 0.52</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>30 min</td>
<td>0.35 ± 0.017</td>
<td>0.22 ± 0.02</td>
<td>0.016 ± 0.0011</td>
<td>8.49 ± 0.37</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>∆ (%)*6</td>
<td>31.4 ± 4.8</td>
<td>39.5 ± 5.3</td>
<td>11.1 ± 1.0</td>
<td>13.6 ± 1.3</td>
<td>—</td>
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* Data are the averages ± standard deviations from three independent incubations. ∆ = [100 × (EC50 (15 min) − EC50 (30 min))] / EC50 (15 min)

REFERENCES


