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Rotating Droplet Hydrodynamic Electrochemistry for Water Toxicity Bioassay Based on an Electron-Transfer Mediator

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Abstract: An electrochemical bioassay based on the rotating droplet electrochemistry by using electron transfer mediator was developed for the evaluation of a wide variety of pollutants such as antibiotics, heavy metals and pesticides in the water environment. Ferricyanide was used as electron transfer mediator for obtaining the catalytic response of *Escherichia coli*. The electrochemical response of *E.coli* was measured via hydrodynamic chronoamperometry in a microdroplet on a screen-printed carbon electrode (SPCE). The constructed electrode system successfully evaluate the catalytic response of *E.coli* solution in presence of ferricyanide. The assay for antibiotic toxicity on *E.coli* was carried out. The EC_{50} for ampicillin, sulfamonomethoxine, chlorotetracycline, tetracycline and oxytetracycline valuated by the pre-incubation method were 0.26, 0.77, 5.25, 18.5, 19.0 μM, respectively. The toxicity order was ampicillin > sulfamonomethoxine > chlorotetracycline > tetracycline>oxytetracycline. The proposed method can be used to evaluate the antibiotic toxicities in different real sample, such as pond water, powder and raw milk. Recoveries were found in the range 90 and 99%. The developed methods do not require additional incubation time to evaluate toxicity.

Keywords: electrochemical bioassay; mediator; *Escherichia coli*; hydrodynamic voltammetry; antibiotics

1. Introduction

Antibiotics are biologically active substances with an extensive use in human and veterinary medicine as therapeutics or growth promoters [1]. A large amount of antibiotic (50 to 90%) are excreted from the body as unchanged form through urine, feces and manure, eventually reaching the water environment through sewage treatment plants, soil amendment or organic fertilizers used in the agricultural fields [2,3]. In addition, antibiotics may directly reach the water environment through the wastes of pharmaceutical plants and hospitals [4]. Thus, antibiotic contamination have commonly been detected in waste water effluents, surface waters, ground waters, sea water and living organism [5–9]. This causes the development of antibiotic resistance genotypes of bacteria [10] and the fatal risk to the indigenous microorganisms [11] and threatened to the ecosystem and human life. In addition, their sustained release to the various environmental compartments produce complex toxicities after mixing with other chemical substances in the environment. Thus there is a great concern in monitoring the overall antibiotic toxicities in the water environment by using appropriate analytical methods. Chemical analysis methods are used to determine the absolute concentration of known single pollutant. However, these methods are not suitable for evaluating the overall toxicities in the water environment.

Consequently, bioassay have been developed to determine the overall toxicities in the environment using fish [12], animal cell, luminescent bacteria [13], algae [14,15] and microorganisms

[16–21]. Microorganisms have been extensively used in bioassay because of their high sensitivity towards toxin, easy manipulation [22–29]. Furthermore, microorganism are a primary producer of the ecosystem and exhibits similar physiological response as higher organisms [30], thus the toxic impact analysis on microorganism help us to know the influence on higher organism by illuminating any small changes in water quality [31,32].

There are many benefits of electrochemical detection techniques in bioassay, such as relatively simple, sensitive, rapid and inexpensive method. In addition, it is applicable to evaluate the toxicities in color sample with suspended solid containing analytes. In several recent works, the electrochemical bioassay using redox mediators have been developed to evaluate the toxicity [33–37]. The mediators participated in the microorganism respiration [38] and accept the electrons from the respiratory chains. Thus, the currents of reduced mediators reflects the microbial activity. In response to a toxicant, the microbial activity is disturbed which can be easily evaluated as a deviation away from the currents produced by healthy cells. This approach enables a rapid and reliable screening for the evaluation of toxicity [34,39].

The aim of this study is to develop a new electrochemical bioassay based on the rotating droplet electrochemistry using an electron-transfer mediator. Ferricyanide was used as electron transfer. The mediator participates in the respiration chain of *Escherichia coli* by accepting the electron in periplasmic space and oxidized at the electrode surface as shown in Figure 1. Thus, the oxidation current of mediator can reflect the microbial activity. In the presence of pollutants, the capacity of the central function of cellular metabolism is disturbed, caused a change of current flow compared to the healthy cells. In this technique which enables the hydrodynamic amperometric analysis in a microliter volume of sample solution, the rotating disk electrode (RDE) exhibits dual functions, such as, it can be used to efficiently blend the sample solution and concurrently acts as an electrochemical detection device. The RDE promotes the convective mass transport to the electrode surface because of the fast rotation speed. As a result, it causes effective mixing that reduces the incubation time for the biochemical reaction. The advantages acquired from RDE detection in a microdroplet (40 μL) reduced the diffusional distance, which results in shorter assay time, rapid detection, and, therefore, faster assay times. In addition, another benefit of using microdroplet is the decrease in dilution of sample, which causes lower detection limits [40,41]. Thus using RDE for detecting the electrochemical reaction in microliter sample solution is of great interest.

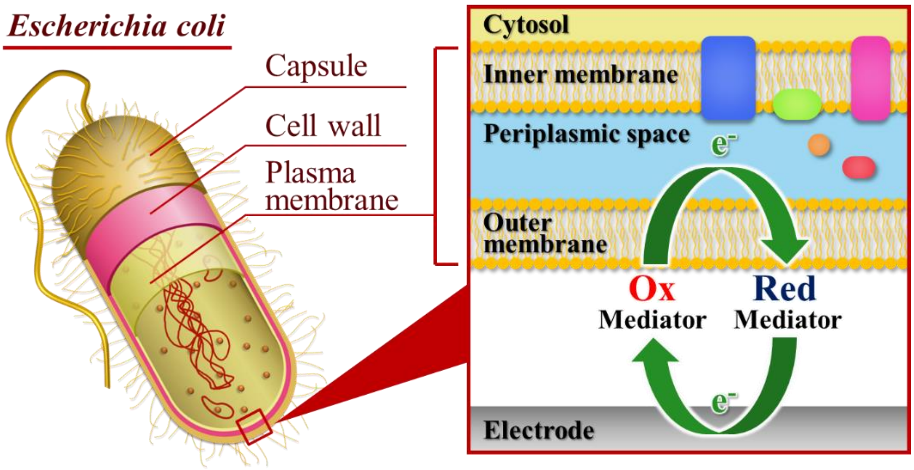

Figure 1. Principle of electrochemical bioassay using mediator.

2. Materials and Methods

2.1. Reagent and Solution Preparation

Potassium hexacyanoferrate (III) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate buffer solution. Ampicillin, tetracycline, sulfamonomethoxine, chloroteracycline, oxytetracycline were obtained from Sigma-Aldrich (St. Louis, MO, USA). The phosphate buffer solution (PBS, pH 7.4) contained 0.044 M NaH_2PO_4, 0.056 M NaH_2PO_4. All reagents used were of analytical grade, and the water was sterile deionized water. Bacto tryptone was purchased from Difco Company (Detroit, MI, USA). The Luria–Bertani (LB: 10.0 g/L bacto tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl) broth was sterilized in high-pressure steam at 120°C for 20 min.

2.2. Culture, Maintenance and Harvesting the E. coli

Escherichia coli K-12 strain was used as a bacterial stain. *E.coli* K-12 was cultured on nutrient agar plates at 4°C, replicated regularly to confirm its viability. A 200 ml solution of autoclaved broth was inoculated with a colony of *E.coli*, then incubated anaerobically at 37°C for 5 hours to reach the stationary phase. Later on, the microorganisms were harvested by centrifuging at 3000 rpm for 30 min and resuspended in PBS. The solution was diluted 10-fold with PBS buffer and checked the absorbance value at 600 nm (OD_{600}) by using UV-2450 UV/Vis detector (Japan spectroscopic company Co., Tokyo Japan). The bacterial suspension was used for the experiments on the day of harvesting.

2.3. Instrumentation and Selection of Electrodes

Hydrodynamic amperometry with a rotating disk electrode (RDE) were carried out using an electrochemical analyzer (ALS-1200, Bioanalytical systems Inc. (BAS) (West Lafayette, IN, USA). The thick-film screen printed electrochemical micro-device printed onto a PVC pad with a size of 3.4×1.0×0.05 cm (DRP-110, Dropsens) and embedded with solvent resistant three electrodes system, like reference, working and counter electrode. The glassy carbon, carbon and Ag/AgCl of screen-printed electrode were used as working, counter and reference electrode, respectively. The box connector (DSC, Dropsens) was used to obtain the electrochemical interface between the RDE, SPCE and the potentiostat. Before starting the measurement, the surface area of the glassy carbon disk electrode was polished, consecutively by adding 0.3 and 0.05 µm alumina paste and later washed well with water.

2.4. Procedures of Experimental Equipment Settings and Electrochemical Detection

The RDE was placed perpendicular to the screen print electrode and contacted at a distance of 2.5 mm. *E.coli* solution (40 µL) was kept in center position between SPCE and RDE. The optimum potential for the ferricyanide (300 mV) was applied to the electrode and the rotation rate was fixed at 3,000 rpm. The assay for antibiotic toxicity on *E.coli* was demonstrated by pre-incubation test method. The measurement was carried out with 40 µL of *E.coli* and toxicant mixture and 30 s later 10 µL mediator solution was added. In this test method, the antibiotic was firstly added during the incubation of *E.coli*. The hydrodynamic chronoamperograms were recorded for 300 s (Figure 2).

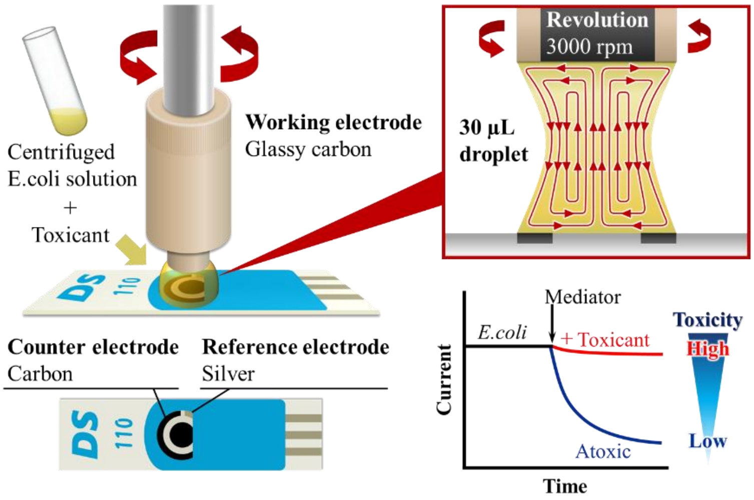

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Figure 2. The experiment set up for the hydrodynamic electrochemistry for water toxicity bioassay using an electron-transfer mediator and the hydrodynamic chronoamperograms expected to be obtained.

3. Results and Discussion

3.1. Investigation of Catalytic Response of *E.coli*

Figure 3 demonstrates the catalytic response of *E.coli* in presence of ferricyanide mediator by using hydrodynamic chronoamperometry. In comparing with baseline current, a sharp increase of response current after adding the ferricyanide (1 mM) with the *E.coli* was observed at 300 s. The obtained current response was increased to 20 µA. This result indicated that ferricyanide were mediated by the catalytic response of *E.coli*. The current response was measured at higher (µA level) even at higher concentration of ferricyanide (45 mM; nA current) reported in other study [42]. This might be due to the high rotation rate of RDE which allows the rapid mass transfer [43] to the electrode surface.

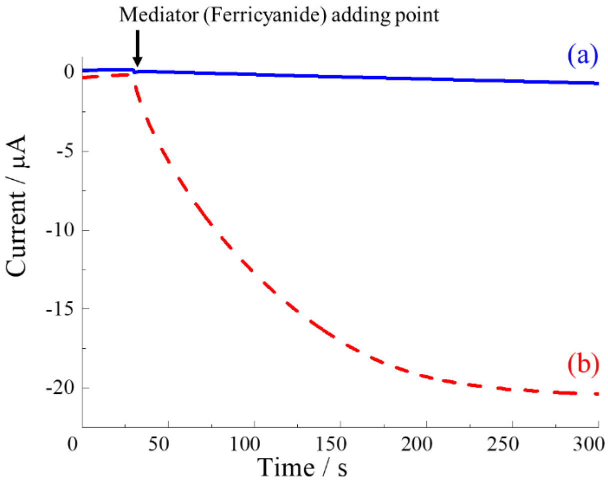

Figure 3. The catalytic response of *E.coli* in presence of ferricyanide mediator by using hydrody-namic chronoamperometry. The hydrodyanamic chronoamperograms for 1 mM ferricyanide with (a) PBS buffer and (b) *E.coli*.

3.2. Effect of *E. coli* on the Electrochemical Detection

The effect of *E.coli* on the electrochemical detection were evaluated by the hydrodynamic linear sweep voltammetry (Figure 4). Fifty µL of droplet including 10 µL of ferrocyanide with *E.coli* or PBS buffer were measured with different rotation rates of 500, 1,000, 1,500, 2,000, 2,500 and 3,000 rpm at a scan rate of 100 mV·s^{-1}. The Levich's equation [40] was used to find the relationship between mass–transfer limited current for oxidation of ferrocyanide and rotation of angular velocity. The equation is as follows,

$$i_l = 0.620nFAC_0D^{2/3}\nu^{-1/6}\omega^{1/2} \quad (1)$$

where i_l is the mass-transfer limited current, n is the number of electrons involved in the reaction, C_0 is the analyte concentration, ν is the kinematic viscosity of the fluid, and ω is the angular velocity of the disk (2π × rpm). The limiting currents showed a positive linear relationship with square root of ω in presence of *E.coli*. This indicates that the electrochemical response of *E.coli* depends on the convective mass transport of mediator resulting from the hydrodynamic flow caused by RDE. The limiting currents also showed a similar positive linear relationship with square root of ω in absence of *E.coli*. This indicates that the electrochemical reaction of mediator is not influenced by the high concentration of *E.coli*. Therefore, *E. coli* did not affect the electrochemical reaction of mediator.

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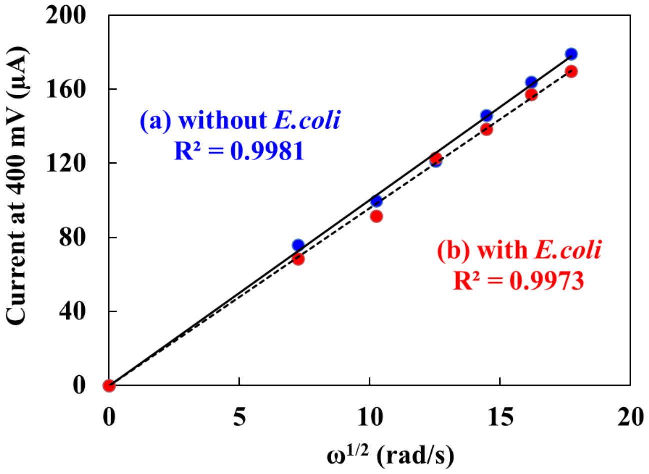

Figure 4. Correlation between the mass–transfer limited current for oxidation of ferrocyanide and rotation of angular velocity ($\omega^{1/2}$). The hydrodynamic linear sweep voltammograms were obtained in micro-droplet solution comprising 10 μL ferrocyanide with 40 μL buffer solution (a) or buffer solution containing *E.coli* (b).

3.3. Effects of Mediator Concentration on E.coli Catalytic Response

Figure 5 shows the results for demonstration of the hydrodynamic chronoamperograms obtained from different concentrations of ferricyanide. Each concentration of ferricyanide (10 μL) was injected to 40 μL of *E.coli* solution. There was an increment of anodic current with increasing the concentration of ferricyanide added into the droplet solution containing *E.coli*. This result was found within only 300 s. The increase of catalytic response of *E.coli* was observed with the increment of concentration of ferricyanide. When the concentration of ferricyanide was increased to 20 mM, there will be more ferricyanide transforming into ferrocyanide and oxidized at the electrode surface. As a result the greatest response current was observed at high concentration of ferricyanide. On the other hand, at minimum concentration of 0.25 mM of ferricyanide, the smallest response current was detected, indicated that the catalytic response of *E.coli* depends on the concentration of ferricyanide which was consistent with a previous report [42].

The hydrodynamic amperometric responses of *E.coli* with different concentrations of ferricyanide were superbly fitted by using Michaelis-Menten equation:

$$v = v_{max}\,[S]/(K_M + [S]) \tag{2}$$

The Michaelis-Menten constant (K_M) and the maximum velocity (v_{max}) acquired from the Hanes-Woolf plots was 0.27 mM at a rotation rate of 3,000 rpm. This clearly indicated that the hydrodynamic amperometry with RDE system is applicable in investigating the catalytic activity of *E.coli* for performing the toxicity test. In addition, the catalytic response of *E.coli* was even observed at a low concentration of ferricyanide.

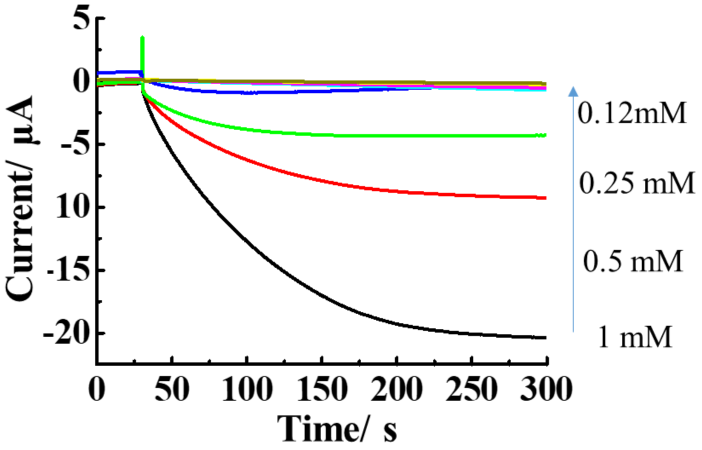

Figure 5. The hydrodynamic chronoamperograms obtained from different concentrations of ferricyanide at 0.12, 03.25, 0.5, and 1.0 mM. Each concentration of ferricyanide (10 µL) was injected to 40 µL of *E.coli* solution (20×10^9 cells/mL). The measurements were performed by the RDE system at 300 mV applied potential with a rotation rate of 3,000 rpm.

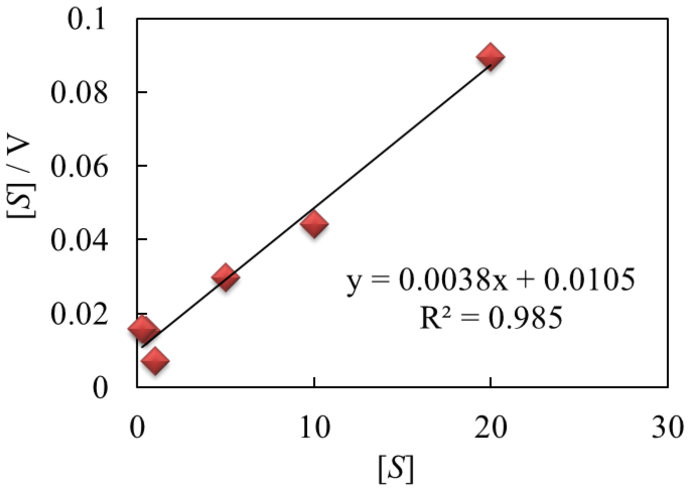

Figure 6. Hanes-Woolf plots of ferricyanide in *E.coli* solution obtained by the RDE system at 300 mV applied potential with a rotation rate of 3,000 rpm.

3.4. Effects of E.coli Concentration on Catalytic Response

The relationship between catalytic response and the concentrations of *E.coli* was investigated as shown in Figure 7. The increase of current mediated by the ferricyanide was observed with the increment of cell densities of *E.coli*. When the cell density of *E.coli* increased to 20×10^9 cells/mL, there will be more *E.coli* to show the catalytic response. As a result, the greatest response current was observed at high call densities of *E.coli*. On the other hand, at minimum cell density of 1.25×10^9 cells/mL of *E.coli*, the smallest response current was detected, indicated that the catalytic current depends on the *E.coli* cell densities. In addition, the proposed method can detected the catalytic response at a low concentration of *E.coli* solution.

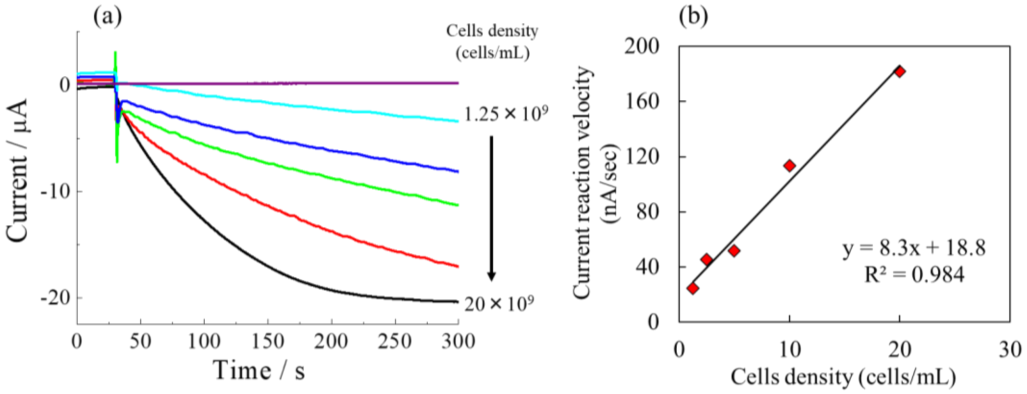

Figure 7. (a) The hydrodyanamic chronoamperograms for ferricyanide exposed to 40 µL *E.coli* with different cell densities of 1.25×10^9, 2.5×10^9, 5×10^9, 10×10^9, 20×10^9 cells/mL The measurements were carried out by the RDE at applied potential, 300 mV. (b) Relationship between the current reaction velocity and the cells density of *E.coli*.

3.5. Evaluation of Toxicities of Antibiotics on E.coli

The dose-response curves of total 5 antibiotics obtained by using the proposed pre-incubation method are shown in Figure 8. Electrochemical response of *E.coli* is decreased resulting a deviation of current flow compared to the unaffected cells. This change of current is considered as the bacterial inhibition to those pollutants. The EC_{50} value for ampicillin, sulfamonomethoxine, chlorotetracycline, oxytetracycline, and tetracycline were calculated as 0.26, 0.77, 5.25, 19.0, and 18.5 µM and the toxicity order was ampicillin > sulfamonomethoxine > chlorotetracycline > tetracycline > oxytetracyclien.

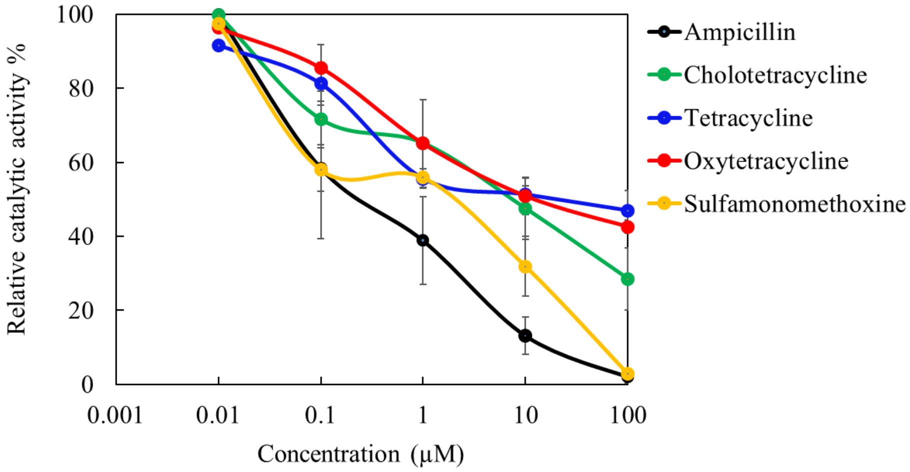

Figure 8. The dose response curves for 5 antibiotics (ampicillin, chlorotetracycline, tetracycline, oxytetracycline, sulfamonomethoxine). The measurements were performed by the RDE system at 300 mV applied potential with a rotation rate of 3,000 rpm.

3.6. Mean Recovery Test

In order to evaluate the possible analytical applications of the proposed toxicity test, various concentrations (1 and 10 µM) of tetracycline was evaluated in different matrixes, such as powder milk, pond water and raw milk, as shown in Table 1. The levels of antibiotics in powder milk, pond water and raw milk were almost similar with the levels of experimental sample, indicated that the proposed method can be usable for the determination of toxicity in real sample. In addition, recovery studies were done for testing the accuracy of the proposed method. In all cases, mean recovery values ranged between 90 and 99%, as shown in Table 1. The value of relative standard deviation was small. These observations showed that the proposed method is precise and reproducible.

Table 1. Recovery study of tetracycline in different matrixes.

Matrix	Additional concentration of tetracycline (μM)	Found concentration (μM)	Recovery (%)	RSD (%)
Pond water	1	0.97	97	2.1
	10	9.61	96	3.8
Milk	1	0.90	90	10
	10	9.02	90	10
Powder milk	1	0.99	99	0.18
	10	9.27	92	7.7

4. Conclusions

The result presented in this study confirm that the electrochemical bioassay based on the rotating droplet electrochemistry successfully evaluate the catalytic response of E.coli in presence of ferricyanide. The pre-incubation test method can evaluate the toxicities of antibiotics in wide variety of real sample with a good accuracy. Because the proposed method did not require additional incubation time, it can evaluate the water toxicity within short period of time.

Author Contributions: Conceptualization, H.K; methodology, K.S. and H.K.; investigation, K.S., A.Y. and H.K.; resources, K.S., K.S. (Kazuharu Sugawara), and H.K.; writing—original draft preparation, A.Y.; writing—review and editing, K.S., K.S. (Kazuharu Sugawara) and H.K.; visualization, K.S., A.Y., and H.K.; supervision, K.S. and H.K.; project administration, H.K.; funding acquisition, H.K. All authors have read and agreed to the published version of the manuscript.

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