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Development of an Online Sulfur-Oxidizing Bacteria Biosensor for the Monitoring of Water Toxicity

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Abstract A toxicity monitoring system based on the metabolic properties of sulfur-oxidizing bacteria (SOB) in continuous and fed-batch modes has been applied for the detection of nitrite (NO_2^--N). In this study, the effects of different concentrations of NO_2^--N (0.1 to 5 mg/L) on the SOB bioreactors were tested. We found that 5 mg/L NO_2^--N was very toxic to the SOB bioreactors in both continuous (R1) and fed-batch (R2) modes, showing complete inhibition of SOB activity within 2 h of operation. R1 and R2 were operated in different ways; however, the EC inhibition and recovery patterns were very similar. The EC rate increased with an increasing NO_2^--N concentration in both continuous and fed-batch modes. The addition of 5 mg/L NO_2^--N in continuous mode decreased the average EC rate by 14.38±2.1 μS/cm/min; while in fed-batch mode, the EC rate decreased by 23 μS/cm/min. Although the toxicity monitoring system could detect 0.5–5 mg/L NO_2^--N, it could not detect 0.1 mg/L NO_2^--N in either continuous or fed-batch operation. Thus, the SOB biosensor method presented is useful to detect toxic agents such as NO_2^--N within a few minutes or hours.

Keywords Biosensor · Nitrite · Sulfur-oxidizing bacteria · Toxicity · Wastewater

Introduction

Nitrite (NO_2^--N), an intermediate in the oxidation of ammonium to nitrate (NO_3^--N), is much more toxic than NO_3^--N to human health and the aquatic environment [1–3]. Accumulation of high concentrations of NO_2^--N in organism may changes hemoglobin to methemoglobin and lead to anoxia or methemoglobinemia in aquatic organisms [2, 4]. In terms of NO_2^--N

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intoxication, aquatic organisms are at higher risk as compared to terrestrial organisms because NO_2^--N in the ambient water can be actively taken up across the gill epithelium and can accumulate to substantially high concentrations in the bodily fluids [2, 3]. Therefore, it is necessary to monitor and remove high concentrations of nitrogen compounds from wastewater before being discharged into natural water bodies to ensure the environmental safety of stream and drinking water [5].

The presence of heavy metals and other toxic chemicals in water has been determined by chemical analysis using techniques such as gas chromatography, atomic absorption spectroscopy, and high-performance liquid chromatography [6, 7]. However, chemical analysis alone is not enough to evaluate the environmental risk of heavy metals and other toxic chemicals and does not give information regarding biological activity [8, 9]. Although chemical analysis is sensitive and quantitative, it is laborious and expensive [5, 9]. Therefore, there is a need to develop inexpensive methods for the assessment of contaminants in water systems.

In recent years, bioassay tests have been used for the assessment of contaminants emerging from contaminated sites or agricultural fields to evaluate the environmental risk of toxic chemicals in microorganisms [5, 7, 8, 10]. Biological toxicity tests detect toxic substances based on a change in physiological characteristics or mortality on representative organisms at different trophic levels [7, 11, 12]. Very recently, a biosensor based on sulfur-oxidizing bacteria (SOB) has been developed and used for the detection of heavy metals and other toxic chemicals in water [5, 7, 10]. Some SOB, such as those of the genus *Acidithiobacillus*, can use elemental sulfur (S°) particles as the electron donor and oxygen as an electron acceptor, as illustrated in Eq. (1) [13]:

$$S^\circ + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+,\ \Delta G^{\circ\prime} = -587\ \text{kJ/reaction} \quad (1)$$

The SOB biosensor exploits the ability of SOB to oxidize sulfur particles under aerobic conditions to produce sulfate (SO_4^{2-}) [10, 13]. SOB oxidize elemental sulfur (S°) to sulfuric acid (H_2SO_4) under aerobic conditions [13]. In the presence of toxic substances, the SOB activity is inhibited, which will cause an increase in pH and a decrease in electrical conductivity (EC) [5, 10]. In this study, a SOB-based toxicity monitoring system in continuous and fed-batch modes was used to assess the toxicity of NO_2^--N by measuring changes in EC.

Materials and Methods

Preparation of Culture and Medium

Aerobic return activated sludge (1 mL) taken from the Chuncheon Wastewater Treatment Plant in Chuncheon City, Gangwondo, Korea, was used to inoculate the sulfur master culture reactor (SMCR). The SMCR consisted of elemental sulfur (S°, 500 g, 2–4 mm in diameter) placed in a 1-L beaker that was filled with 500 mL of dilute synthetic stream water. After incubation for 5 days, the reactor was operated in fed-batch mode (waste all the water, feed 500 mL synthetic stream water). Synthetic stream water was prepared by diluting the following nutrient mineral buffer (NMB) solution 100 times: 3.13 g/L ($NaHCO_3$), 0.31 g/L (NH_4Cl), 0.13 g/L (KCl), 4.22 g/L (NaH_2PO_4), and 2.75 g/L (Na_2HPO_4). Trace metal and vitamins solutions were also diluted 100 times and added to the synthetic stream water as previously described [14]. The SMCR was aerated using a stone diffuser with a flow rate of 150–250 mL/min and incubated at 30 °C. The pH, EC, and alkalinity of the synthetic stream water were 6.9±0.14, 0.12± 0.014 mS/cm, and 45 mg/L as $CaCO_3$, respectively.

Construction of Reactors and Operation

Three identical cylindrical reactors were constructed as described elsewhere [10, 15] for toxicity monitoring. A schematic diagram of the toxicity monitoring system is shown in Fig. 1. The online toxicity monitoring system (OTMS-2013) was constructed by M-Cubic Co., Ltd. Daejeon, Korea, with dimensions of 50 cm(W)×66 cm(D)×115 cm(H). The operating temperature of the toxicity system was 30–40 °C. The three SOB bioreactors (R1, R2, and R3) were packed with 50 mL (82 g) of S° particles from the SMCR, and air was introduced from the bottom of the reactor at a flow rate of 100–200 mL/min.

Continuous Mode The R1 bioreactor was fed synthetic stream water continuously in up-flow mode using peristaltic pumps as described elsewhere [5, 7, 10]. It was operated at a hydraulic retention time of 30 min for 1–2 days to reach steady-state conditions. The biosensors were then fed with 0.1–5 mg/L NO_2^--N as the toxic substance, and the effluent EC was monitored.

Fed-Batch Mode For fed-batch tests, the procedures followed are described elsewhere [16]. Briefly, the SOB biosensors (R2 and R3) were fed synthetic stream water semicontinuously (60 mL/min; for R2, 1 min of rapid feeding and 30 min of batch reaction; for R3, 1 min of rapid feeding began 15 min after the batch reaction of R2 had begun, followed by 30 min of batch reaction) in up-flow mode using adjustable peristaltic pumps. Upon reaching stead-state conditions (i.e., stable EC and pH values), 0.1–5 mg/L NO_2^--N (in the form of $NaNO_2$) was applied to the SOB biosensor influents, and the resultant effluent EC value was monitored. One cycle consisted of 1 min of rapid feeding and 30 min of reaction time.

Chemicals and Analyses

All reagents were of analytical grade and were used without further purification during the experiment. NO_2^--N (in the form of $NaNO_2$, Sigma-Aldrich, St Louis, MO, USA) was used as

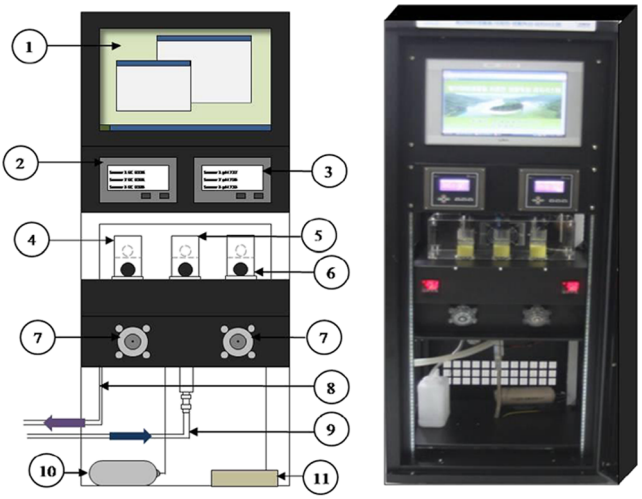

Fig. 1 Schematic diagram of the toxicity monitoring system: (*1*) screen, (*2*) pH meter, (*3*) EC meter, (*4*) R1, (*5*) R2, (*6*) R3, (*7*) peristaltic pump, (*8*) effluent line, (*9*) influent line, (*10*) air pump, (*11*) LAN

the toxic chemical in the experiments. NO_2^--N was added to the influent and the toxicity was monitored by measuring changes in the EC. The EC value was measured using a meter constructed from an electrode supplied by M-Cubic Cooperation, Daejeon, Korea. The EC meter was connected to a personnel computer and all data were automatically recorded every 2 min.

Results and Discussion

Typical Results of the Toxicity Monitoring System

Typical results obtained from the toxicity monitoring system used in this study are shown in Fig. 2. Three SOB bioreactors were operated, in which the R1 bioreactor was operated in continuous mode, while the R2 and R3 bioreactors were operated in fed-batch modes, respectively. A stable EC value of ~1.134 mS/cm was maintained in the SOB bioreactor (R1) fed with synthetic stream water. In R2, during the 1 min of rapid feeding, the EC value was maintained at ~0.601 mS/cm; while during the 30-min reaction time, it was ~1.138 mS/cm. Similarly in R3, during the 1 min of rapid feeding, the EC value was maintained at ~0.380 mS/cm; while during the 30-min reaction time, it was ~0.819 mS/cm. Toxic material was added in the influent line of the system as shown in Fig. 1 after a stable EC value was maintained. After the addition of toxic material (NO_2^--N) to the SOB bioreactors, the EC decreased to the level of the influent EC in R1. In R2 and R3, the EC also decreased. Next, the toxicity monitoring system was operated without toxic substance addition using synthetic stream water. When the synthetic stream water was added, the EC increased in all three reactors.

Continuous Tests of NO_2^--N

The effect of different concentrations of NO_2^--N (0.1–5 mg/L NO_2^--N) on SOB activity in the bioreactors in continuous mode is shown in Fig. 3a. As soon as NO_2^--N was fed into the bioreactor (R1), a decrease in EC occurred within minutes to a few hours, depending on the concentration of the toxicant. NO_2^--N was very toxic at 5 mg/L. When 5 mg/L NO_2^--N was

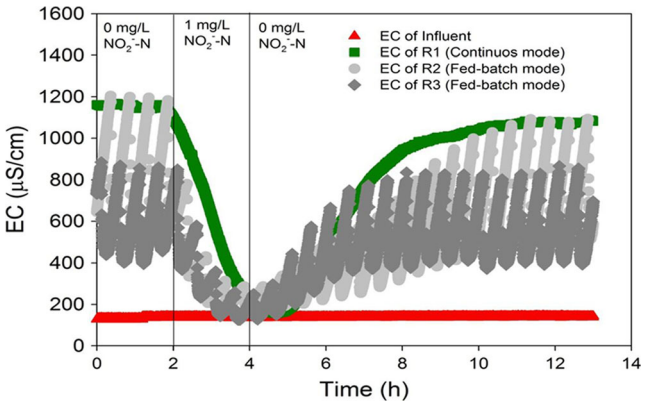

Fig. 2 Typical results of the toxicity monitoring system (R1: continuous mode, R2 and R3: fed-batch mode, NO_2^--N model toxicant)

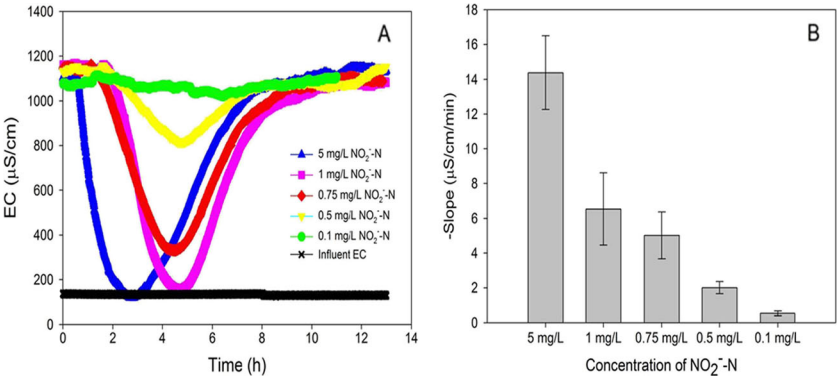

Fig. 3 **a** The effect of different NO_2^--N concentrations on the SOB bioreactor in continuous mode, and **b** slope changes after NO_2^--N addition

added to the SOB bioreactor, the effluent EC decreased from 1.134 to 0.810 mS/cm in 30 min. The effluent EC decreased to the influent EC (0.130 mS/cm), i.e., the SOB activity was completely inhibited. After complete inhibition, the SOB bioreactor was operated without the toxicant to resume its original function. The SOB bioreactor was fed with synthetic stream water and resumed its original function after operation for approximately 5 h.

Generally, the percentage of inhibition was decreased as the concentration of NO_2^--N added to the SOB bioreactor decreased. When 1 mg/L NO_2^--N was added to the SOB bioreactor, the effluent EC did not decrease beyond 0.155 mS/cm (influent EC=0.134 mS/cm). When the effluent EC reached its lowest value of 0.155 mS/cm, the bioreactor was operated without the addition of NO_2^--N for 4.13 h so that it could resume its original function, i.e., the active EC value was resumed. The addition of 0.75 or 0.5 mg/L NO_2^--N to the SOB bioreactors resulted in a decrease of the effluent EC. When 0.5 mg/L NO_2^--N was added to the bioreactor, the final effluent EC did not decrease beyond 0.812 mS/cm. The SOB bioreactor did not detect 0.1 mg/L NO_2^--N, as no change in EC was observed after the injection of 0.1 mg/L NO_2^--N to the bioreactor.

As the concentration of the toxicant was decreased, the steepness of the EC slope also decreased (Fig. 3b). From the slope-intercept calculation, it was observed that the addition of 5 mg/L NO_2^--N to the bioreactor decreased the average EC rate by 14.38±2.1 µS/cm/min. The addition of 1 mg/L NO_2^--N or 0.75 mg/L NO_2^--N to the bioreactor decreased the average EC rate by 6.5±2.07 and 5.02±1.3 µS/cm/min, respectively. When 0.5 mg/L NO_2^--N was added to the bioreactor, the average EC rate decreased by 2.01±0.35 µS/cm/min. Although the addition of 0.1 mg/L NO_2^--N to the bioreactor did not significantly changes the effluent EC, the average EC rate decreased by 0.53±0.14 µS/cm/min in continuous mode.

Fed-Batch Tests of NO_2^--N

In fed-batch mode, NO_2^--N was added to the bioreactors (R2 and R3) after the EC reached steady-state conditions. The effect of different concentrations of NO_2^--N (0.1–5 mg/L NO_2^--N) on SOB activity in fed-batch mode is shown in Fig. 4. The EC rates were greater with 5 mg/L NO_2^--N and 1 mg/L NO_2^--N as compared to 0.75 mg/L NO_2^--N and 0.5 mg/L NO_2^--N. Figure 5 shows the effect of different NO_2^--N concentrations in relation to the EC rate in fed-batch mode (R2).

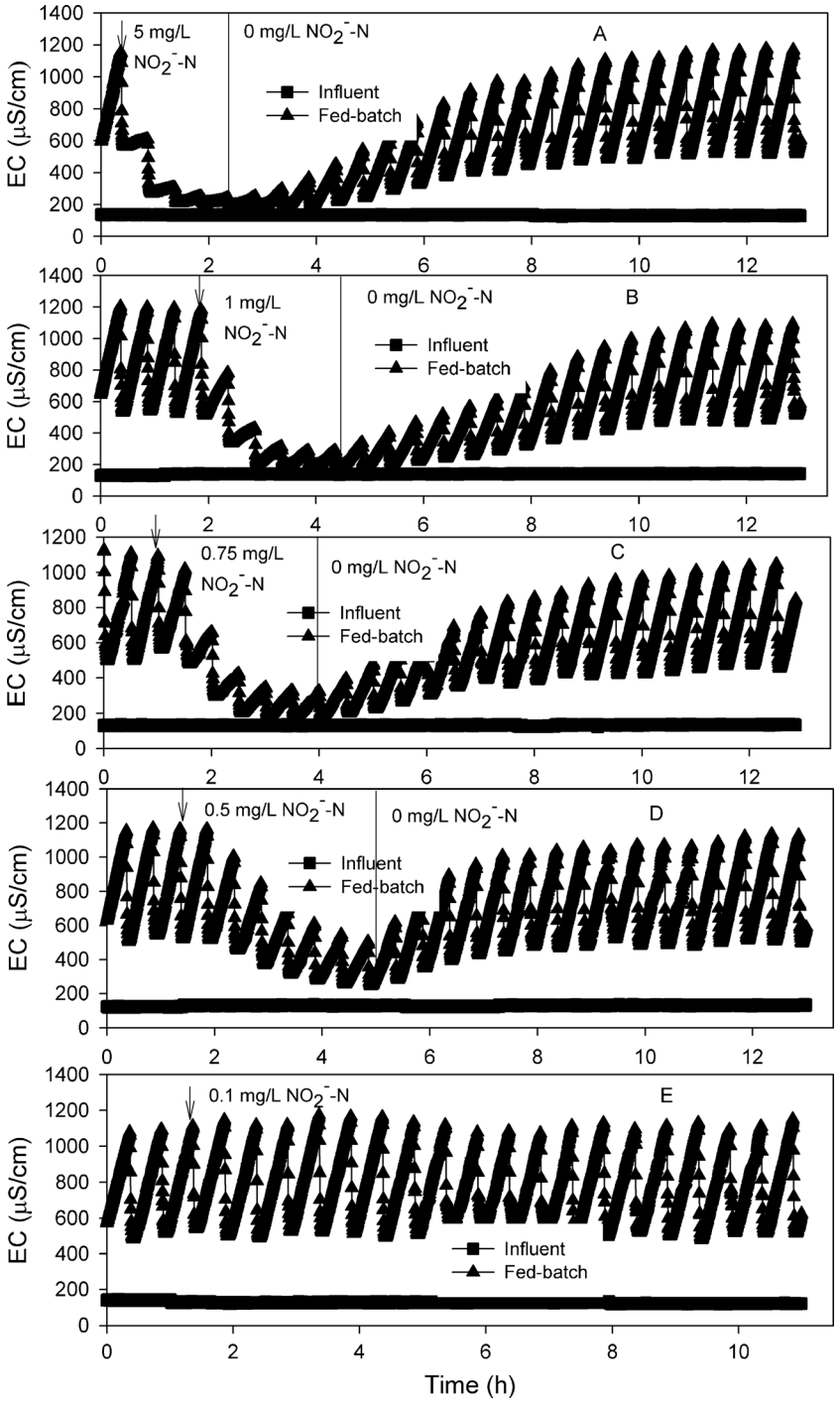

Fig. 4 The effect of different NO_2^--N concentrations on the SOB bioreactor in fed-batch mode

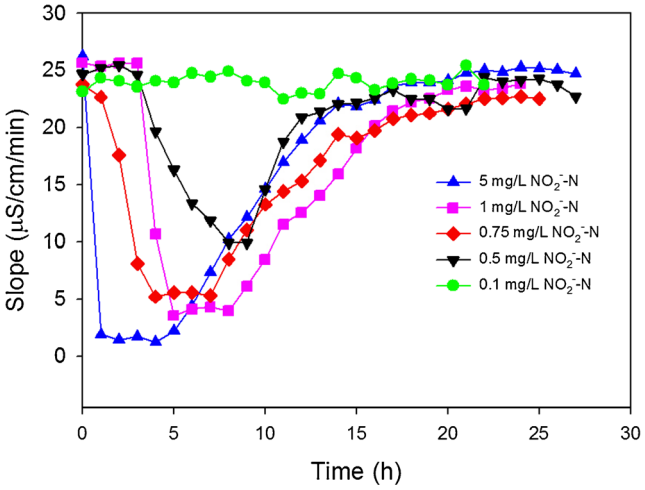

Fig. 5 The effect of NO_2^--N on the EC rate in fed-batch mode

The relationship of NO_2^--N concentrations to SOB activity is expressed by the EC rate (Fig. 5). As soon as 5 mg/L NO_2^--N was added to the SOB bioreactor (R2), the increasing effluent EC rate decreased immediately during the 30-min reaction time. When 5 mg/L NO_2^--N was added to R2, the EC rate decreased from 25 to 2 μS/cm/min. Based on the EC slope calculation, 92 % of the SOB activity was inhibited after the addition of 5 mg/L NO_2^--N to R2. When 1, 0.75, or 0.5 mg/L NO_2^--N was added to R2, the lowest EC rate was obtained after two or three cycles. The addition of 1 mg/L NO_2^--N to R2 decreased the EC rate from 25 to 4 μS/cm/min after two cycles (84 % of the SOB activity was inhibited). When 0.5 mg/L NO_2^--N was added to R2, the EC rate decreased from 25 to 10 μS/cm/min after five cycles and 60 % of the SOB activity was inhibited.

Daphnia and fish are widely used as a test organism in acute and chronic toxicity tests of chemicals and complex effluents [17, 18]. Gross et al. [19] evaluated the toxicity of NO_2^--N on juvenile *Litopenaeus vannamei* (white shrimp) and reported a half maximal effective concentration (EC_{50}) of 8.9 mg/L NO_2^--N after exposure for 96 h. Similarly, *Ceriodaphnia dubia* were

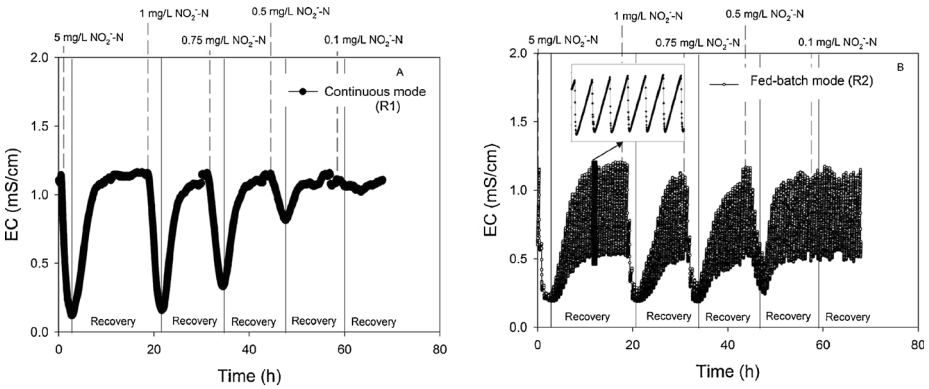

Fig. 6 Comparison of R1 and R2 results

exposed to NO_2^--N and the 6-day EC_{50} was reported to be 2.4 mg/L NO_2^--N [18]. In another toxicity test, *Pimephales promelas* (fathead minnows) were exposed to NO_2^--N and the 32-day EC_{50} was reported to be 14.6 mg/L NO_2^--N [18]. However, using *Daphnia* or fish as the test organism requires a long incubation period. In this regard, the SOB biosensor has some merit over these bioassays. As shown by the present study, the SOB biosensor method is better than these other methods as the toxicity of NO_2^--N can be detected within a few minutes or hours.

Comparison of Continuous and Fed-Batch Mode

Although R1 was operated in a different way compared to R2 and R3, the inhibition and recovery patterns were very similar. Figure 6a, b shows the recovery of EC values in the toxicity monitoring system in both continuous (R1) and fed-batch modes (R2). When a higher concentration of toxicant (5 mg/L NO_2^--N) was added to the SOB bioreactor in continuous mode, the effluent EC decreased to the influent level in approximately 2 h of operation. Similarly, after the addition of 5 mg/L NO_2^--N to R2, the effluent EC decreased to the minimum value and the SOB activity was completely inhibited within 2 h. After complete SOB inhibition, both bioreactors (R1 and R2) were operated without toxicant and the EC increased. The recovery period was slightly longer in the fed-batch mode as compared to the continuous mode.

Conclusions

In this study, the toxicity monitoring system was operated in both continuous and fed-batch modes for the detection of NO_2^--N in water. The important conclusions are highlighted below.

1. The toxicity monitoring system could detect 0.5–5 mg/L NO_2^--N, but not 0.1 mg/L NO_2^--N, in both continuous and fed-batch mode operation.
2. The EC rate increases with increasing NO_2^--N concentration in continuous mode. Under higher NO_2^--N concentrations, the rate was greater as compared to the data at 0.1 mg/L NO_2^--N.
3. With fed-batch mode, when 5 mg/L NO_2^--N was added to the bioreactor, the EC rate decreased by 23 μS/cm/min and 92 % of the SOB activity was inhibited.
4. After SOB inhibition by NO_2^--N, the SOB activity of the bioreactor was able to recover in a short period of time (~2 h).
5. Other toxic chemicals such as Cr^{6+}, Cd^{2+}, and Pb^{2+} also showed similar EC trend (data not shown).

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