Bioresource Technology 102 (2011) 4196–4203

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Acclimation and activity of ammonia-oxidizing bacteria with respect to variations in zinc concentration, temperature, and microbial population

Seungyong Lee, Kyungjin Cho, Juntaek Lim, Woong Kim, Seokhwan Hwang *

School of Environmental Science and Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, South Korea

ARTICLE INFO

Article history:
Received 5 October 2010
Received in revised form 6 December 2010
Accepted 7 December 2010
Available online 15 December 2010

Keywords:
Ammonia-oxidizing bacteria
Zinc
Temperature
Response surface analysis
Acclimation

ABSTRACT

Activity of ammonia-oxidizing bacteria (AOB) to simultaneous variation in Zn^{2+} concentration (0.01–3.5 mg/L), temperature (23–33 °C), and AOB concentration (3–30 × 10^6 gene copies/mL) in a steel industry wastewater treatment plant was evaluated. Two equations were developed to describe the lag period (i.e., AOB acclimation) and ammonia oxidation rate (i.e., growth of the AOB) depending on the variables. AOB concentration and temperature both had significant effects on lag period and the ammonia oxidation rate. Zn^{2+} concentration only had a significant effect on ammonia oxidation rate at 5% α-level. There was a significant interaction between AOB concentration and temperature for both lag period and ammonia oxidation rate. The effects of the variables were not significant when AOB concentration was higher than 2.0 × 10^7 copies/mL. There was no visible shift or changes in AOB communities based on DGGE analysis with *amoA* gene primers.

1. Introduction

Conventional integrated steel manufacturing primarily consists of three unit processes: reduction of iron ores to steel in a blast furnace, casting, and rolling. A large volume of cooling water is required in the processes and is inevitably contaminated with various materials (Kim et al., 2008). Major pollutants in the wastewater generated from steel manufacturing include suspended solids, metallic compounds, oil and grease. The waste streams also include gasification products such as ammonia. The wastewater creates a serious disposal problem for the steel processing industry due to its large discharge amount as well as its heterogeneous characteristics (Lay-Son and Drakides, 2008; WSA, 2009).

A biological nitrogen removal (BNR) system employing autotrophic nitrification followed by heterotrophic denitrification is currently an essential step in the overall management of wastewater generated in steel manufacturing industries due to the tightened effluent discharge limits in many nations (Kelly et al., 2004; Wyffels et al., 2004). Regardless of the type of BNR process, the overall treatment efficiency is primarily dependent on two different kinds of microbial activities in the nitrification phase: oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB); and the subsequent oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB).

The first step of nitrification (i.e., ammonia oxidation) is generally regarded as a rate-limiting step due to the low growth rate of AOB and their high susceptibility to changes in growth conditions. Autotrophic nitrifying bacteria (nitrifiers) cannot acclimate as readily to the inhibitory event as can heterotrophic denitrifying bacteria (denitrifiers). This difference in adaptive capacity might render the overall nitrification process sensitive to changes in environmental conditions (Limpiyakorn et al., 2005).

In most BNR processes, a long sludge retention time (SRT), usually at least 10 days (Campos et al., 2007; Hao et al., 2009), is required due to the low growth rate of AOB. Consequently, the residual ammonia concentration in the system is likely to be very low. When an external supply of the limiting substrate (i.e., ammonia) for growth and/or energy is not available or is extremely low, microorganisms turn to internal substrate sources. For example, if certain enzyme proteins used in metabolizing the original exogenous organic substrates are no longer needed, then they may become substrates themselves and are primarily used to maintain cells in a viable state. When ammonia concentration is low during the ammonia oxidation process, AOB in such a system can be considered as being in a maintenance energy dominating stage (Price and Sowers, 2004; Vogeler et al., 2008). AOB under this condition are likely to be stagnant in growth and are also highly susceptible to variations in environmental factors such as temperature, pH, and presence of heavy metals (Limpiyakorn et al., 2005).

Zinc (chiefly Zn^{2+}) is a heavy metal frequently found in wastewater generated from steel manufacturing using oxygen furnaces and has non-biodegradable properties. Depending on the concentration of Zn^{2+} and the length of exposure with AOB in wastewater, zinc causes an acute or chronic effect by binding to the active site of ammonia monooxygenase (AMO). This displaces the functional

* Corresponding author. Tel.: +82 54 279 2282; fax: +82 54 279 8299.
E-mail address: shwang@postech.ac.kr (S. Hwang).

0960-8524/$ - see front matter Crown Copyright © 2010 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.12.035

copper ligand and thus results in inhibition of AOB growth (Hu et al., 2004; Radniecki et al., 2009). For example, *Nitrosomonas europaea* exposed to 3.4 μM $ZnCl_2$ showed a 61% decrease in the rate of nitrite production and a 69% decrease in the ammonia dependent specific oxygen uptake rate (AMO-SOUR) following 30 min of exposure (Park and Ely, 2008). *Nitrosococcus mobilis* exposed to 10 μM $ZnCl_2$ showed an immediate decrease in activity and did not exhibit noticeable recovery, resulting in a 100% decrease in nitrite production rates and an 86% decrease in AMO-SOUR (Radniecki and Ely, 2008). Thus, inhibition of growth of AOB by various heavy metals has been of great interest to researchers (Hu et al., 2002; You et al., 2009).

It must be noted, however, that the degree of microbial toxicity is likely to depend on the interaction between the inhibitor(s) and other environmental factors, which might be stimulatory for microbial growth and thus possibly complement the inhibitory effect. It has been reported, for example, that the mixed liquor volatile suspended solids (MLVSS) level in a bioreactor treating industrial wastewater is an important factor in the resistance to zinc and copper toxicity (Sirianuntapiboon and Hongsrisuwan, 2007). The addition of more sludge to methanogenic pure cultures can also significantly increase the tolerance of the microorganisms to zinc, copper, and nickel (Jarrell et al., 1987). It could be suggested, therefore, that the inhibitory effects of heavy metals could be alleviated by increasing the biomass concentration of the system. In this manner, the ratio of the inhibitor to the quantity of AOB can be lowered. A lower ratio would improve the tolerance of AOB to inhibitory compounds in the treatment system.

Temperature is also one of the most influential factors on the growth of nitrifying bacteria. Nitrifiers are temperature sensitive and, consequently, so is the rate of nitrification. The rate of nitrification falls sharply at lesser than 10 °C but increases almost directly proportionally with increasing temperature over the range of 10–30 °C. This activity curve fits with evidence showing the optimal temperature for nitrification in the BNR process is around 30 °C in the absence of inhibitor(s) (Gerardi, 2002). Therefore, it would be expected that the rate of nitrification would accelerate with increasing temperature and, conversely, that the rate would significantly decline with decreasing temperature at a given Zn^{2+} concentration.

The main objective of this research was to develop predictive equations for a mechanistic understanding of the AOB activities (i.e., acclimation and growth) in steel industry wastewater treatment plants by incorporating the simultaneous effects of Zn^{2+}, temperature, and AOB concentration into a response surface analysis (RSA). Such models could be used to formulate more reliable technological guidelines and to serve as the basis for technical decisions in wastewater treatment plant operation.

Molecular assays targeting 16S rRNA and *amoA* genes of AOB were used for the microbial quantification and community structure analysis, respectively. The results from this work could be used as general guidelines on the capacity of heterogeneous AOB populations to accommodate expected magnitudes of disruption and recovery times, and, where possible, controllable factors that may affect the response.

2. Methods

2.1. Inoculum system and seed preparation

A continuous stirred tank reactor (CSTR) with 7 L working volume was set up to produce a consistent seed to inoculate the subsequent batch cultures for RSA. The reason for using the inoculum CSTR was to minimize any confounding effect associated with the use of inconsistent inoculum. Ammonia-containing wastewater (i.e., 300 mg NH_4^+-N/L) obtained from a local steel-processing industry was used as a substrate for the inoculum CSTR, which was operated at 10 days hydraulic retention time (HRT) and 28 °C. pH and dissolved oxygen (DO) concentration was controlled to be above 7.5 and 2.0 mg DO/L, respectively. The steady state effluent containing no detectable NH_4^+-N concentration (data not shown) was collected for centrifugation at 14,000*g* for 5 min when needed. The supernatant was discarded and the pellet was washed with 20 mM $NaHCO_3$ to remove residual ammonia. After another centrifugation and washing step, the concentrated seed was prepared and aerated until it was used as the inoculum for the subsequent batch trials. Steady state was assumed when the concentration of the NH_4^+-N,NO_2^--N,*and*NO_3^--N did not vary by more than 10%.

2.2. Experimental design and batch RSA tests

The RSA experiment was designed with a central composite cube design (Lee et al., 2008; Myers and Montagomery, 1995) and batch ammonia oxidation reactions were conducted at each condition (Table 1). The ranges of each independent variable were determined based on the results from monthly sampling of the full-scale treatment plant for 1 year before the RSA experiment.

Each trial was performed with a modified ISO 9509 method (Do et al., 2008). Concentrated (tenfold) synthetic wastewater was initially prepared with 2.65 g/L $(NH_4)_2SO_4$ for a final concentration of 56 mg/L NH_4^+-N. The concentrated solution also contained 16.8 g/L $NaHCO_3$ as buffering agent, which could maintain the pH at 7.5 during ammonia oxidation. A 100×-concentrated $ZnCl_2$ stock solution, the concentrated seed, and the synthetic wastewater were added to 250 mL flasks, with distilled deionized water (DDW) to a total working volume of 125 mL. The experimental conditions were shown in Table 1. The flasks were kept in a shaking incubator, stirred at 130 rpm, at the desired temperature of 23, 28, or 33 °C. The cultures were periodically sampled until the ammonium ion was completely depleted.

2.3. Real-time quantitative PCR

The AOB concentration in the inoculum effluent was measured using LightCycler 480 Real-time PCR System (Roche Diagnostics,

Table 1
Experimental design and observed lag periods and ammonia oxidation rates.

Trial	Independent variables			Dependent variables	
	Zn^{2+} concentration (mg/L)	AOB concentration (10^6 copies/mL)	Temperature (°C)	Lag period (h)	AOR[b] (NH_4^+-Nmg/L/h)
1	0.01	3	23	72	0.7
2	3.5	3	23	141	0.6
3	0.01	30	23	1.2	4.7
4	3.5	30	23	0.5	3.5
5	0.01	3	33	26	0.4
6	3.5	3	33	22.4	0.2
7	0.01	30	33	0.4	9.4
8	3.5	30	33	0.9	7.0
9[a]	1.755	16.5	28	0.8 ± 0.01	3.4 ± 0.01
10	0.01	16.5	28	1.8	4.9
11	3.5	16.5	28	0.2	2.1
12	1.755	3	28	35.6	0.2
13	1.755	30	28	0.7	7.3
14	1.755	16.5	23	1.4	2.2
15	1.755	16.5	33	2.7	6.4

[a] Center point. The experiment was repeated 3 times and the response represented average values.
[b] AOR, ammonia oxidation rate.