Improvement of Ammonia Oxidation in Wastewater Using Rockwool as a Carrier in Wastewater Treatment

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*1 Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8572, Japan

Abstract

Improvement of microbial ammonia oxidation method using a rockwool as a carrier in wastewater treatment was examined through batch and continuous operation. In batch operation, a nitrifying bacterium, *Nitrosomonas europaea* (NBRC 14298), and phosphorus and iron-complexes were highly attached to the rockwool. In continuous operation, the operation with rockwool (RW) was shortened the hydraulic retention time (HRT) from 20 h to 10 h, and the maximum ammonia removal ratio was 87.5%. The average of ammonia removal ratio was performed 73.1%, 78.2% at 20 h and 10 h, respectively, at aeration rate of 3000 ml·min⁻¹. The ammonia oxidation rate was 22.3 mg-N·g⁻¹-carrier⁻¹·h⁻¹ when the ammonia loading rate was 25.5 mg-N·g⁻¹-carrier⁻¹·h⁻¹. Quantitative PCR results revealed that around 10¹⁵ cells·g⁻¹-carrier were immobilized to the RW. Therefore, the RW could retain a large number of nitrifying bacteria, improve the degree of nitrification and have widely application.

Keywords: Ammonia-oxidizing bacteria, Real-Time PCR, ammonia oxidation rate, Rockwool

1. Introduction

The removal of nitrogen compounds from wastewater, such as agricultural, industrial and municipal wastewater, is essential for an aqueous environment, and is usually performed in a nitrification step and a denitrification step by microorganism. In the wastewater treatment, the nitrification step is a rate-limiting step because of the slow growth rate of the pertinent bacteria, such as *Nitrosomonas europaea*, which permits their wash-out from wastewater treatment reactor. Chemo-lithotrophic nitrification is a two-step process, consisting of the oxidation of ammonia to nitrite, which is in turn oxidized to nitrate (Kowalchuk, G. A. and Stephen, J. R., 2001). Ammonia oxidation is thought to be the initial rate-determining step in nitrification (Kowalchuk, G. A. and Stephen, J. R., 2001). For the efficient removal of nitrogen, a long retention time of such nitrifying bacteria is important factor for the continuous operation. Thus, improvement of ammonia oxidation, the rate and the ratio using a carrier for immobilized bacterial cells has been studied; entrapment in a gel matrix of polyvinyl alcohol (Cao, G. et al., 2002), immobilization of porous cellulose (Matsumura, M. et al., 1997), porous polyurethane (Jun, H. B. J. et al., 2000) and various materials (Wijffels, R. H. and Tranmper, J., 1989, Ginkel, C. G. et al., 1983, Sumino, T. et al., 1992, Sumino T. et al., 1992) has been reported.

However, it was speculated that these carriers are high cost, non recycling-oriented material and hard to get in developing country. Thus, authors focused on a rockwool (RW), which is made from rock and is less expensive and recycling oriented material. Although the RW is widely used in hydroponic culture, but it was
taken high cost to discard RW. In wastewater treatment, the RW is used methane fermentation (Maekawa, T., 1998, Yang, Y. et al., 2004). The methane production rate with RW as a carrier was performed 4.5 times than no carrier in the reactor at 5°C in methane fermentation (Maekawa, T., 1998). In methane fermentation, temperature control use low energy with the RW as a carrier. In contrast, few studies have examined nitrogen removal in wastewater treatment using RW as a carrier (Matsuoka, T. et al., 2000, Matsuoka, T., 2001, Doshu, N. et al., 1993, 1994).

In this study, authors examined improvement of ammonia oxidation in batch operation for demonstrating bacteria immobilization on the RW and quantitative analysis of bacteria attached on the RW as a carrier in continuous operation.

2. Materials and Methods

*Nitrosomonas europaena* (*N. europaena*, NBRC 14298), an ammonia oxidizing bacterium, was used in this study. Cells were cultured and immobilized under aseptic condition. The bacteria were grown in shake flask with HEPES Medium (NBRC Medium No. 829) at 28°C. Inorganic synthetic wastewater, supposing domestic wastewater (Park, K. et al., 2000) was formulated from the following ingredients per l of distilled water: 97.4 mg NH₄Cl, 1,500 mg KH₂PO₄, 500 mg NaHCO₃, 300 mg NaCl, 5 mg CaCl₂·2H₂O, 500 mg MgSO₄·7H₂O, 5 mg Fe₃(OH)₃, 0.1 mg Na₂MoO₄·2H₂O, 2 mg MnSO₄·5H₂O, 0.2 mg CuSO₄·5H₂O, 0.01 mg ZnSO₄·7H₂O. The pH was adjusted 7.5-8.0 with 1 M NaOH.

Rockwool (Nittobo, Japan), cut in 10 mm cube, was used in this study. The RW was washed with 99% acetone, dried, washed with ultra-pure water, and then dried again. Table 1 shows the composition of RW.

<table>
<thead>
<tr>
<th>Composition (Nittobo)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂</td>
<td>43%</td>
</tr>
<tr>
<td>CaO</td>
<td>33%</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>15%</td>
</tr>
<tr>
<td>MgO</td>
<td>6%</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>MnO</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>specific gravity</td>
<td>2.48</td>
</tr>
<tr>
<td>bulk density</td>
<td>0.08</td>
</tr>
<tr>
<td>porosity</td>
<td>&gt; 90%</td>
</tr>
<tr>
<td>pH</td>
<td>1.5</td>
</tr>
<tr>
<td>mean of fiber diameter</td>
<td>5 µm</td>
</tr>
</tbody>
</table>

2.1 Batch operation

2.1.1 The experimental apparatus

Four 2 l Erlenmeyer flasks contained 2 l of the inorganic synthetic wastewater were set up: the sterile control, a reactor without a carrier, and reactors containing the RW at the ratio of 5% and 10% of the working volume, respectively. All four reactors were sterilized for 40 min at 121°C after the RW added, then they were inoculated with *N. europaena* (final population density, 10⁶ cells·ml⁻¹), which only sterile control was added through sterile filtration with Millex-GV (pore size 0.22 µm) and incubated for 3 days under aerobic condition with slow stirring. All reactors were run under 30°C in the dark. Sterile air with filter (0.2 µm) was used for aeration. The initial aeration rate was 400 ml·min⁻¹, and then was adjusted according to the volume of the synthetic wastewater to maintain a constant flow rate per volume of the inorganic synthetic wastewater.

2.1.2 Analytical methods

The optical density (O.D.) of inorganic synthetic wastewater was determined by the spectrophotometer (UV-1200, Shimadzu, Japan) at 660 nm. The NH₄-N concentration of inorganic synthetic wastewater was determined by colorimetric method (Japan water works association, 1993).

2.2 Continuous operation

2.2.1 The experimental apparatus

The scheme of the experimental apparatus was shown in Fig. 1. The reactor was up flow bioreactor. To
immmobilize the cells on the RW before operation started, the inoculated N. europaea (initial population density, 10^7 cells·mL⁻¹) was grown in the inorganic synthetic wastewater in the reactor for 10 days. The RW was then used as a carrier in continuous operation. The fluidized bed reactor (1 l active volume) was set up with 10% RW of active volume and inoculated with bacteria (final population density, 10^9 cells·mL⁻¹), and was maintained as follows condition for 390 h. The initial aeration rate was 200 ml·min⁻¹, and was adjusted to maintain the dissolved oxygen (DO) concentration above 7 mg·L⁻¹. The pH was controlled 8.0 ± 0.3 using 1 M NaOH and HCl. The cultivation temperature was controlled 29 ± 1°C with a water jacket. The inorganic synthetic wastewater was mixed with magnetic stirrer at 500 rpm. The initial HRT was set to 20 h. After the inorganic synthetic wastewater was run over 3 times of 20 hours HRT, stability of the removal NH₄-N ratio was confirmed. Then, the HRT was adjusted to 10 h.

### 2.2.2 Analytical method

NH₄-N and NO₂-N concentrations were analyzed by colorimetry (Japan water works association, 1993). NO₃-N concentration was analyzed by hydrazinium sulfite reduction method. (Saijyo, Y. and Mitamura, O., 1995) The number of bacteria immobilized in the RW was determined by quantitative PCR method using the real-time PCR (7500 Real-Time PCR System, Applied Biosystems Japan, Tokyo, Japan) from the number of copies of 16S rRNA gene which N. europaea has one copy in a cell (Chain P. et al., 2003). A new primer set was designed using Primer Express software (Applied Biosystems, U.S.A.) (Table 2). The DO concentration was determined by a DO meter. The pH was determined by a pH meter.

### Table 2. Primers used in PCR experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>816*F</td>
<td>TTGTCCGATCTAATTTACGTAGG</td>
</tr>
<tr>
<td>916*R</td>
<td>TCCCCGTCATATCTCCTTGTGAG</td>
</tr>
</tbody>
</table>

F: forward primer; R: reverse primer
*: The position is based on the 16S rRNA gene of N. europaea.

### 2.2.3 Extraction of DNA from the RW and Real-Time PCR method

After 390 h of continuous operation, the RW was cut into pieces (approximately 0.03 g wet weight) and the DNA of N. europaea in attached the carrier was extracted by modifying the freeze-thaw (Miller, D. N. et al., 1999) and nonenzymatic method (Sharma, A. D. A. and Singh, J., 2005). Standard N. europaea samples containing 10^5-10^9 cells·mL⁻¹ were prepared for real-time PCR in consideration of DNA extraction efficiency.

Real Time PCR was carried out by SYBR® Premix Ex Taq™ (Perfect Real Time; Takara Bio, Japan) and 0.2 μM of 816F and 916R primers according to the manufacture’s instructions.

### 3. RESULT and DISCUSSION

#### 3.1 Batch operation

The population density of planktonic bacteria in the inorganic synthetic wastewater was decreased only in the RW reactors (Fig. 2). Bacterial population density in
the reactor with 5% RW was less than that in the reactor with 10% RW and the density in the former also declined faster than that in the latter. Therefore, the RW in the former reactor might have had a larger contact area for bacteria than the latter. Fig. 3 indicates fluorescent of a large amount of attached *N. europaea*, and self-fluorescence of the RW. These results showed that the RW was suitable for immobilizing *N. europaea*.

Time course of O.D.660 in inorganic synthetic wastewater was shown in Fig. 4. The O.D. clearly decreased in the reactor with RW compared with the reactor without RW. The color of inorganic synthetic wastewater changed from turbid white to transparent. The same result was seen in the sterilized control with RW (data not shown). This result indicated that RW attached the phosphorus and iron complex efficiently, which responsible for the turbidity. This ability might allow RW to capture sludge.

Time course of the NH₄-N concentration in each reactor is shown in Fig. 5. The gradients shows ammonia oxidation rate. The rate was calculated without the removal by aeration from sterile control. The initial ammonia oxidation rates were 0.75 mg-N·h⁻¹ in the reactor without RW, 0.31 mg-N·h⁻¹ in the reactor with 5% RW, and 0.41 mg-N·h⁻¹ in the reactor with 10% RW. The ammonia oxidation rate was the highest in the reactor without RW because *N. europaea* would be greater direct contact with ammonia.

*N. europaea* was fast immobilized on the RW carrier though operation period was short (72h) (Fig. 3).
Actually in wastewater treatment reactors, a short HRT is required for the treatment of a large volume of wastewater. However, since the growth rate of *N. europaea* is very slow, a short HRT will cause wash-out. Therefore, a stable population of bacteria, as achieved by the RW, is critical. For this reason and ammonia oxidation rate, authors used a reactor with 10% RW in the continuous operation experiment.

### 3.2 Continuous operation

#### 3.2.1 Reactor performance

Time course of planktonic bacterial population density in the inorganic synthetic wastewater with 10% RW was shown in Fig. 6. Time course from 40 h to 390 h of inorganic nitrogen (NH₄-N, NO₂-N and NO₃-N) concentrations in the inorganic synthetic wastewater with 10% RW, pH and DO concentration were shown in Fig. 7 and 8, respectively. The planktonic bacterial population density decreased with time. At an HRT shorter than the doubling time of *N. europaea*, the cells immobilized on the RW dominated in the reactor. The NH₄-N removal ratio averaged 92.1% in the first 120 h operation, while the NO₂-N concentration decreased, the aeration rate adjusted to 300 ml·min⁻¹ at 60 h and 400 ml·min⁻¹ at 120 h. However, NH₄-N concentration increased from 120 h to 260 h, and NO₂-N and NO₃-N concentrations were decreased. Therefore, HRT was up to 40 h for the recovery of cell activity because of HRT (40 h) longer than the doubling time of *N. europaea*. As a result, NH₄-N concentration was clearly decreased and NO₂-N, NO₃-N and planktonic bacterial population density were clearly increased (Fig. 6, 7). *N. europaea* can oxidize NH₄-N to NO₂-N to NO₃-N under aerobic condition, and can reduce NO₂-N to NO and N₂O only under anaerobic to micro-aerobic condition or high cell densities (Remde, A. and Conrad, R., 1990). RW particle flow was become worse since the particles were attached other particles formed clumps at the bottom of the reactor. It was considered that the low NO₂-N concentration and high NO₃-N concentration up to 220 h showed that the immobilized cells did not take oxygen enough. Through this ability might prevent the RW from wash-out, the aeration rate was up to 3,000 ml·min⁻¹ at 290 h to allow the immobilized cells to take oxygen and to improve the particle flow. When the aeration rate was 400 ml·min⁻¹, the carrier flow motion was worse for forming clumps with experimental time. However, the
carrier flow motion was improved at the aeration flow rate 3,000 ml·min⁻¹.

The HRT adjusted 20 h again from 290 h. After this operation, NH₄-N concentration was decreased from 310 h, and the mean of its concentration was 22.0% of total nitrogen from 310 to 350 h. NO₂-N concentration was increased from 310 h, and the mean of its concentration was 68.8% of total nitrogen from 310 to 350 h. NO₃-N concentration was general constant concentration, and the mean of its concentration was 9.2% of total nitrogen from 310 to 350 h. Though the total nitrogen concentration was not balanced out at 290 and 310 h, these indicate that N. europaea converted NH₄-N to NO₂-N to NO₃-N. The low NH₄-N concentration was retained for 3 times HRT, thus, the HRT was reduced from 20 h to 10 h at 350 h of operation. This change kept the NH₄-N concentration low and stable for 3 times HRT. From 290 h to 390 h, the maximum ammonia removal ratio was 87.5%. And the average of ammonia removal ratio was performed 73.1% at 20 h and 78.2% at 10 h. The maximum ammonia oxidation rate was 22.3 mg-N·l⁻¹·carrier⁻¹·h⁻¹ when the ammonia loading rate was 25.5 mg-N·l⁻¹·carrier⁻¹·h⁻¹. There are many studies about the ammonia removal ratio, ammonia oxidation rate and the number of immobilized cells on other carriers in activated sludge or pure culture of bacteria. Table 3 shows the comparison of this study and various researches. In this study, high ammonia removal ratio was performed, while the ammonia oxidation rate was lower than those with other carriers except PVA (Wijffels, R. H. and Tramper, J., 1989, Sumino, T. et al., 1992, Cao, G. et al., 2002, Sumino, T. et al., 1992, Matsumura, M. et al., 1997, Jun, H. B. J. et al., 2000). Jun, H. B. J. et al. reported that the number of ammonia oxidation bacteria and ammonia oxidation rate were positively proportional (Jun, H. B. J. et al., 2000). Therefore, in this study, it was considered that RW have been attained possible higher ammonia oxidation rate for high the number of immobilized cells. However, Jun, H. B. J. et al. and Matsumura, M. et al. determined immobilized cell number by most probable number (MPN) method (Matsumura, M. et al., 1997, Jun, H. B. J. et al., 2000). Since this method was performed by cultivation method, it is reasonable to suppose that the number of immobilized cells is underestimated. The number of total bacteria generally was higher than that of living bacteria (Kogure, K. et al., 1979, 1980, Yu, F. P, et al., 1993, Wery, N. et al., 2006). Though, in this study, real time PCR method counted the number of total bacteria, immobilized cell was more than that of other studies. It was considered that real time PCR method have weaker bias, related DNA loss at DNA extraction and PCR efficiency, than MPN method. Thus, it was seemed that MPN method not suitable for the strict count of the.
immobilized cell number. Authors suggest that quantitative PCR should be used instead of MPN method for the estimating the number of immobilized cells.

Matsumura, M. et al. reported that the pore size of the carrier did not influence to the ammonia oxidation rate, but the carrier size did (Matsumura, M. et al., 1997). This indicated that as the carrier is smaller, the ammonia oxidation rate become higher. Hence, it might be increased the ammonia oxidation rate by reducing the size of RW and increasing the aeration, or by increasing the oxygen partial pressure and stimulating the efficient uptake of oxygen by the immobilized cells, and making better the carrier flow motion.

The results of batch and continuous operation proved that RW could be used as a carrier for nitrifying bacteria in the wastewater treatment. It could retain

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**Table 3. Comparison of various carriers**

<table>
<thead>
<tr>
<th></th>
<th>rock wool (10 mm cubic)</th>
<th>porous cellulose (1 mm cubic)</th>
<th>porous cellulose (5 mm cubic)</th>
<th>porous polyurethane (15 mm cubic)</th>
<th>PVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of immobilized cell</td>
<td>4.01 × 10^{15}</td>
<td>1.74 × 10^{12}</td>
<td>3.30 × 10^{13}</td>
<td>1.82 × 10^{9}*i</td>
<td>N. D.</td>
</tr>
<tr>
<td>(cells·l^{-1}-carrier)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum ammonia oxidation rate</td>
<td>22.3</td>
<td>700</td>
<td>300</td>
<td>55.6*i</td>
<td>18</td>
</tr>
<tr>
<td>(mg-N·l^{-1}-carrier·h^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum ammonia loading rate</td>
<td>25.5</td>
<td>1000</td>
<td>1042</td>
<td>54.9</td>
<td>20</td>
</tr>
<tr>
<td>(mg-N·l^{-1}-carrier·h^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum ammonia removal ratio (%)</td>
<td>87.5</td>
<td>100</td>
<td>80</td>
<td>83.3</td>
<td>90</td>
</tr>
<tr>
<td>HRT (h)</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td>Aeration rate (ml·min^{-1})</td>
<td>3000</td>
<td>Depending on DO*4</td>
<td>Depending on DO*4</td>
<td>1500</td>
<td>Depending on DO*4</td>
</tr>
<tr>
<td>Temperature in reactor (°C)</td>
<td>29 ± 1</td>
<td>25</td>
<td>25</td>
<td>21 ± 2</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>DO Con. (mg·l^{-1})</td>
<td>7.3 (mean)</td>
<td>6</td>
<td>6</td>
<td>&gt; 6</td>
<td>3-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mean)</td>
<td>(mean)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (mean)</td>
<td>8.0</td>
<td>8.0*3</td>
<td>8.0*3</td>
<td>6.4-7.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Experimental time (h)</td>
<td>390 (630*2)</td>
<td>6,600</td>
<td>6,600</td>
<td>1,920</td>
<td>1,440</td>
</tr>
</tbody>
</table>

\*1: Aerobic zone in the carrier. \*2: Including the pre-incubation for the cell immobilization on the RW before operation started. N. D.: No determination. \*3: pH was controlled. \*4: DO concentration was controlled by oxygen gas and nitrogen gas.
bacteria at high concentration, and in the continuous operation could shorten HRT and attach nutrients, such as phosphorus and iron-complex. Since the RW could be used in anaerobic wastewater treatment also, operating costs could be reduced by standardization of the RW carrier in wastewater treatment reactors. Furthermore, the RW is made from common rock, which can be obtained at low cost and waste material from hydroponic culture. The RW would make an efficient carrier not only in developing countries, but also in developed countries, since reusing waste material from hydroponic culture. Therefore, it was indicated that the RW can apply various agricultural structure, such as wastewater treatment from livestock, hydroponic culture and domestic wastewater for preventing aquatic environment from eutrophication.

4. Conclusion

1) High number of N. europaea was immobilized on the RW and RW had a high ability to attach phosphorus and iron-complex. In the short HRT (10 h), the RW was immobilized $10^{15}$ cells·l$^{-1}$·carrier.
2) The maximum ammonia oxidation rate was 22.3 mg-N·l$^{-1}$·carrier·h$^{-1}$ when the ammonia loading rate was 25.5 mg-N·l$^{-1}$·carrier·h$^{-1}$.

Acknowledgements

The authors greatly thank associate professor Hideaki Maseda (Institute of technology and science, University of Tokushima) for good discussion and advising molecular technique.

Reference


廃水処理におけるロックウール担体を用いた
アンモニア酸化の向上

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要 旨

廃水処理における回分操作と連続操作によりロックウールを担体とした微生物のアンモニア酸化法の向上について検討した。回分操作において硝化細菌である Nitrosomonas europaea (NBRC 14298) と鉄とリンの複合体がロックウールに極めて多く付着した。連続操作において、ロックウールを担体とすることで水理学的滞留時間 (HRT) を 20 h から 10 h へと短縮することができ、最大アンモニア除去率は 87.5% であった。曝気流量が 3000 ml・min⁻¹ である時、平均アンモニア除去率は、HRT が 20 h では 73.1%、HRT が 10 h では 78.2% であった。アンモニア負荷速度が 25.5 mg-N·l⁻¹·carrier⁻¹·h⁻¹ の時に最大アンモニア酸化速度 22.3 mg-N·l⁻¹·carrier⁻¹·h⁻¹ となった。定量 PCR の結果、ロックウールに固定された供試細菌は、10¹² cells·l⁻¹·carrier 程度であった。以上より、ロックウールは、多くの硝化細菌を保持することができ硝化速度の向上に寄与することがわかった。

キーワード：アンモニア酸化細菌、Real-Time PCR、アンモニア酸化速度、ロックウール