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Dinitrogen production from ammonia by *Nitrosomonas europaea*

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Abstract

In this study, the conversion efficiency and optimal conditions of successive nitrification and denitrification were assessed for *Nitrosomonas europaea*, which uses ammonia monooxygenase (AMO), nitrite reductase and nitrous oxide reductase. In aerobic conditions, the first step of nitrification (i.e. $NH_4^+ \rightarrow NO_2^-$) occurred; under oxygen-limiting or oxygen free-conditions, however, there was denitrification, whereby nitrous oxide and dinitrogen were produced from nitrite (NO_2^-). About 7% of the total ammonium was converted to dinitrogen following the successive nitrification and denitrification by *N. europaea*. During nitrification and denitrification, the optimal pH range for the production of nitrite and dinitrogen was found to be 7.0–8.0. A low partial oxygen pressure or oxygen-free conditions were favorable for the production of dinitrogen.

Keywords: Nitrification; Denitrification; Dinitrogen; *Nitrosomonas europaea*

1. Introduction

The high nitrogen level in wastewater has become a growing concern, which has increased the necessity to develop efficient N-removal techniques. Undesirable nitrogen compounds can be converted through two biological processes: aerobic nitrification (conversion of NH_3 to NO_2^- or NO_3^-) and anaerobic denitrification (conversion of NO_2^- or NO_3^- to N_2) [1,2]. However, in addition to these processes, a range of new microbial processes has recently been reported to occur in wastewater treatment plants. These include aerobic denitrification and heterotrophic nitrification [3,4], anaerobic ammonium oxidation [4] and denitrification by autotrophic nitrifying bacteria [5,6]. These observations were unexpected, as denitrification has previously been regarded as strictly an anoxic process performed by chemoorganotrophic bacteria. Therefore, there has been much effort devoted to developing denitrification systems that employ chemoorganotrophs, despite some experimental disadvantages. In the chemoorganotropic systems, strong efforts are needed to spatially separate oxic, lithoautotropic nitrification from anoxic, heterotropic denitrification [7]. Other inconveniences are: (a) one or more additional substrates are often necessary to achieve complete denitrification [8], since chemoorganotrophic denitrifiers need an organic electron donor; (b) an additional aerobic step is needed for the removal over supplied electron donors. In contrast to this requirement for electron donors extrinsic to the denitrification reactant, ammonia-oxidizers of the genus *Nitrosomonas* (lithoautotropic nitrifying bacteria) such as those found in sewage sludge

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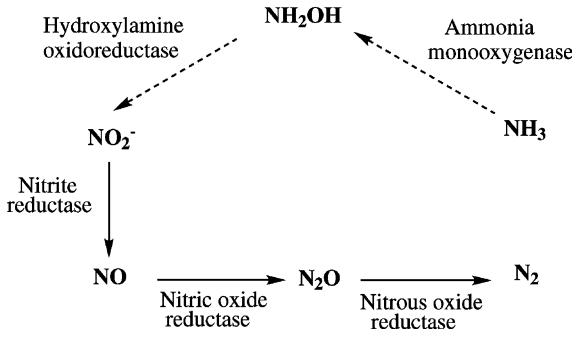

Scheme 1. Pathway of nitrification and denitrification carried out by *N. europaea*. - - - - → Nitrification; ——→ denitrification.

seem to be capable of both nitrification and denitrification by using ammonia as the electron donor (Scheme 1). The ability to both nitrify and denitrify makes *Nitrosomonas* attractive candidates for application in the removal of ammonia nitrogen from wastewater.

Nitrosomonas bacterial cells gain their energy by oxidizing ammonia to nitrite in a two-step reaction [9]. The initial oxidation of ammonia yields the reductant hydroxylamine. This initial reaction is O_2-dependent and is catalyzed by ammonia monooxygenase (AMO). Subsequently, hydroxylamine is oxidized to nitrite catalyzed by hydroxylamine oxidoreductase (HAO). Two of the four electrons generated from the hydroxylamine oxidation are used to support the oxidation of additional ammonia molecules, while the other two electrons enter the electron transfer chain and are used to support CO_2 reduction and ATP biosynthesis [9].

If optimal conditions for these processes can be defined, these nitrifiers/denitrifiers may be employed for N-waste removal in controlled conditions. This potential application is superior to current systems, as it would eliminate the large-scale separation of nitrification and denitrification that is restricting the efficiency of current bacterial N-removal systems.

Therefore, to initiate development of *Nitrosomonas*-governed waste control method, the goal of this work was to quantitate dinitrogen conversion of ammonia by a pure culture of *Nitrosomonas europaea*. To this end, the concurrent nitrification and denitrification carried out by *N. europaea* was monitored.

2. Materials and methods

2.1. Organism

The experiments were performed with *N. europaea* (ATCC 25978).

2.2. Medium

N. europaea were grown lithoautotrophically in ATCC medium 1573 of the following composition: $(NH_4)_2SO_4$ (1.7 g), $CaCl_2{\cdot}2H_2O$ (20 mg), $MgSO_4{\cdot}7H_2O$ (200 mg), FeEDTA (1 mg), K_2HPO_4 (15 mg), trace elements[1] (1 ml) and distilled water (1 l). The medium contained 360 mg/l ammonium nitrogen (NH_4^+–N), and was sterilized at 120 °C for 20 min.

2.3. Growth

Batch cultures of *N. europaea* (ATCC 25978) were grown in 300 ml flasks (200 ml medium) while being stirred at room temperature. Flasks were aerated continuously by an air pump. Air was sterilized through a 0.2 μm filter unit (Millex-FG50, Millipore). During bacterial growth, the pH of the culture solution was maintained at 7.5–7.8 using a peristaltic pump with 10% K_2CO_3. After three days, 200 ml cultures were used to inoculate 550 ml of new medium. Cells were harvested during late logarithmic growth by centrifugation (6762 × *g* for 10 min) 7 days after inoculation. The resulting cell pellet was washed with an ammonium-free medium and then resuspended in a 1:1 ratio dilution of the cell pellet ammonium-free medium. The diluted cells were stored at −80 °C.

2.4. Oxidation of ammonium and formation of nitrite

The oxidation of ammonium and formation of nitrite by *N. europaea* were observed in 300 ml capacity conical flasks containing 200 ml of buffering solution. The pH values of the solutions ranged from 6.0 to 8.0 according to treatment conditions. An amount of 50 mM MES–KOH buffer was used to establish pH values between 6.0 and 6.5, and 50 mM phosphate

[1] $Na_2MoO_4{\cdot}2H_2O$ (10 mg), $MnCl_2{\cdot}4H_2O$ (20 mg), $ZnSO_4{\cdot}7H_2O$ (10 mg), $CoCl_2{\cdot}6H_2O$ (0.2 mg), $CuSO_4{\cdot}5H_2O$ (2 mg) and distilled water (100 ml).

buffer was used for the pH values between 7.0 and 8.0. The concentration of ammonium nitrogen (NH_4^+–N) in the sample solutions was 350 mg/l. The cell density (*N. europaea*) in the solutions was 2.2–3.02 mg-wet cell/ml. The oxygen concentration in the solution was 7.3 mg/l. Flasks were supplied air or O_2 continuously. The flow of air was maintained at 1.25 l/min and that of oxygen at 550 ml/min. Air and oxygen were sterilized through a 0.2 μm filter unit (Millex-FG50, Millipore). Ammonium oxidation and nitrite formation were monitored over time from the beginning of the incubation to 52 h. Solutions were then withdrawn by syringes, filtered with a Millex-GP filter unit (0.22 μm pore size) and analyzed for ammonium and nitrite levels. In control condition, the ammonium oxidation was observed without *N. eruopaea*.

2.5. N_2 formation from ammonia

In aerobic conditions, ammonium was converted to nitrite, while under O_2-limiting or O_2-free conditions, there was successive denitrification, whereby the nitrite was changed to nitrous oxide and dinitrogen gas. The ability of *N. europaea* to produce dinitrogen was tested using batch-type reactors. The batch reactors employed 160 ml serum bottles with white rubber septums. The serum bottles were filled with 50 ml of 52 h incubated solutions of *N. europaea* (the same solutions mentioned in Section 2.4).

In another experiment, the serum bottles were first filled with 25 ml of ammonium-free ATCC 1573 medium and then sterilized at 120 °C for 20 min. Twenty-five millilitres of 52 h incubated solutions of *N. europaea* were added aseptically to the serum bottles such that the total volume of the reaction mixture was 50 ml. Anaerobic conditions were made by substituting O_2 with helium gas (99.99% pure). In aerobic condition, oxygen concentration in the reaction solution was maintained at 0.6 mg/l. Serum bottles were then incubated in a thermostatic water bath incubator at 30 °C. Gas samples were then taken from the headspace with a gas tight syringe and analyzed by gas chromatography.

2.6. Analytical procedure

Nitrite concentrations were determined colorometrically [10] or by HPLC (Inertsil ODS-3, 4.6 mm × 250 mm) in 5.0 mM tetrabutyl-ammoniumhydrogensulfate in 10 vol.% (v/v) methanol, at a pH of 6.4 [11] and a flow rate of 1.0 ml/min. The samples were visualized with a UV–VIS detector (UV–VIS detector SPD-10AV Shimadzu, Japan, wavelength 225 nm). The ammonium concentration was determined colorometrically [10]. Nitrogen gas levels were determined by gas chromatography using a Shimadzu (Kyoto, Japan) gas chromatograph (Model GC-8AIT) equipped with a thermal conductivity detector. Helium was used as the carrier gas. The chromatographic column was stainless steel (3 m × 2 mm) packed with a 13X molecular sieve. Biomass (*N. europaea*) was determined by UV–VIS spectrophotometer (Hitachi U-2000) with the absorbance at 660 nm.

3. Results and discussion

The oxidation of ammonium (i.e. formation of nitrite) by *N. europaea* was observed in a flask containing 200 ml of buffer solution with an ammonium nitrogen (NH_4^+–N) concentration of 350 mg/l. When 2.2 mg-wet cell/ml of *N. europaea* in phosphate buffer (pH 7.5) were incubated with a continuous supply of air or oxygen, NH_4^+–N in 200 ml solution was oxidized to NO_2^-–N at 47 and 37% conversion efficiencies, respectively, in 52 h (Fig. 1). When the starting cell density in the 200 ml solutions was increased, higher NO_2^-–N production in the solution was observed (data not shown), thus indicating that complete consumption of ammonium by *N. europaea* is indeed possible. However, since lower NO_2^-–N conversion efficiency values were observed in the O_2 supply sample, all other nitrification processes were carried out with a continuous supply of air unless otherwise indicated.Next, the production of NO_2^-–N from NH_4^+–N was observed at different pH values. The pH range in which optimal NO_2^-–N production by *N. europaea* was achieved was found to be 7.0–8.0 (Fig. 2). This slightly basic pH range is not surprising since ammonia oxidation is not favorable at acidic pH due to equilibrium shifts away from the NH_3 substrate of AMO to higher NH_4^+ concentration [12]. Interestingly, ammonium consumption and nitrite formation did not occur at a 1:1 ratio. This is likely because some ammonium is utilized by cells for their internal metabolism, and some is changed into hydroxylamine. No ammonium

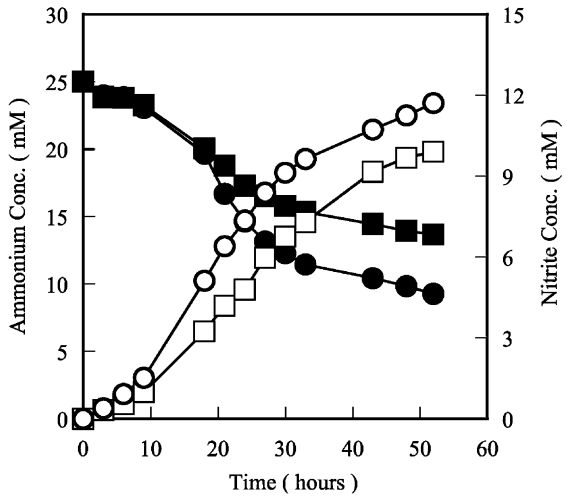

Fig. 1. Ammonium consumption and nitrite formation over time by *N. europaea* in the presence of a continuous supply of air and oxygen. *N. europaea* cells were suspended in 200 ml of a solution containing 50 mM of phosphate buffer (pH 7.5), and 25 mM ammonium chloride. The cell density in the solution was 2.2 mg-wet cell/ml. Air and oxygen flow rates were maintained at 1.25 ml/min and 550 ml/min, respectively. Air supply incubation shows higher ammonium consumption and more nitrite formation than oxygen supply incubation. The symbols (■) and (●) represent the ammonium consumption in oxygen and air supply solutions, respectively. The symbols (□) and (○) represent the nitrite formation in oxygen and air supply solutions, respectively. All reactions were carried out at room temperature.

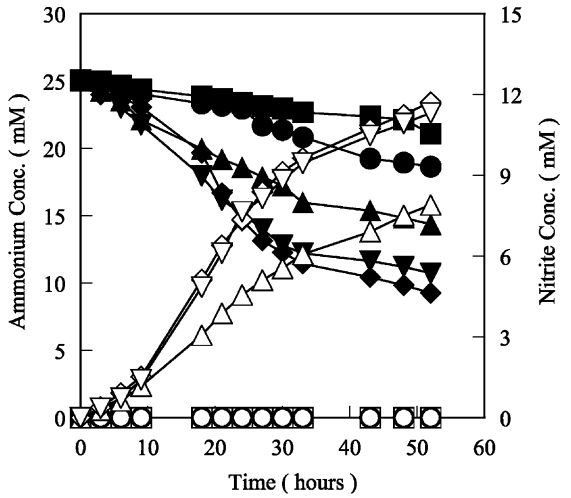

Fig. 2. Effects of pH on the ammonium consumption and nitrite formation by *N. europaea* in the presence of continuous air supply. *N. europaea* cells were suspended in 200 ml of a solution containing 50 mM MES buffer (pH 6.0–6.5) and 50 mM phosphate buffer (pH 7.0–8.0), and 25 mM ammonium chloride. The cell density in the solution was 2.2 mg-wet cell/ml. Flow rate of air was maintained at 1.25 ml/min. Higher ammonium consumption was observed at pH (7.0–8.0). Lower ammonium consumption was observed at acidic pH (6.0–6.5). The symbols (■), (●), (▲), (◆), and (▼) represent ammonium consumption at pH values of 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. The symbols (□), (○), (△), (◇) and (▽) represent nitrite formation at pH values of 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. Each reaction was carried out at room temperature.

oxidation was observed without *N. europaea*. Thus, ammonium stripping was not there at the pH value and aeration condition used.

To observe the denitrification capacity of *N. europaea*, 50 ml of 52 h incubated *N. europaea* solution (the air-supplied samples where oxidation of ammonium to nitrite had already taken place) were transferred to gas-tight serum bottles and anaerobic conditions were generated by helium gas substitution. Under these conditions, total NH_4^+–N was converted to dinitrogen at 2.57% efficiency in 9 days (Fig. 3). Similarly, in samples where ammonium oxidation to nitrite had taken place under a continuous O_2-supply, all the NH_4^+–N converted to dinitrogen at conversion efficiency of 2.34% (Fig. 3). When the oxygen concentration in the reaction solution was 0.6 mg/l, dinitrogen production could occur (data not shown) while it was inhibited at saturated oxygen concentration.

Denitrifying activities of well-studied ammonia-oxidizers commonly include the production of NO, N_2O and N_2 from the reduction of NO_2^- under low O_2 pressure. Consistent with this idea that low O_2 pressure is favorable for dinitrogen production, no dinitrogen production occurred (data not shown) under saturated oxygen concentration conditions, whereas it was observed under low partial O_2 pressure as well as in O_2-free conditions. In *N. europaea*, the enzymes responsible for denitrification are a copper-containing nitrite reductase with cytochrome oxidase activity [13–15], and a nitrous oxide reductase [16,17]. Since, it has been found that the reducing enzymes of *N. europaea* are active and able to reduce nitrite under low partial O_2 pressure, it is likely that *N. europaea* was responsible for the conversions [14,16]. During aerobic denitrification, dinitrogen was detected as an end product, whereby ammonium apparently served

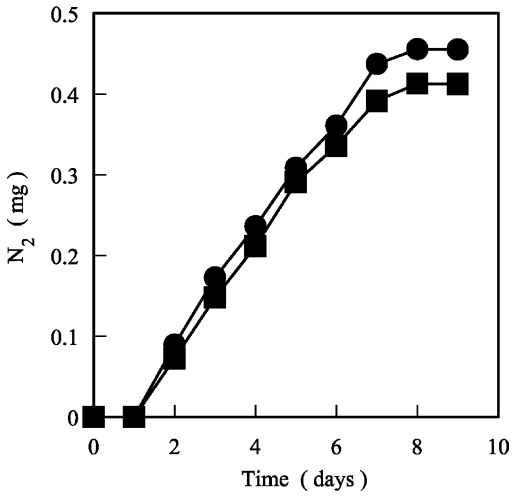

Fig. 3. Dinitrogen production from air and oxygen-supplied samples during nitrification (ammonium oxidation and nitrite formation). The serum bottles contained 50 ml of the solution described in the Fig. 1 caption. Almost the same amount of dinitrogen was observed from solutions supplied with air and with oxygen. The symbol (■) represents the dinitrogen production during denitrification by the previously oxygen-supplied cells. The symbol (●) represents the dintrogen production during denitrification by the previously air-supplied cells. All reactions were carried out in anaerobic conditions at 30 °C. Helium gas was used to replace O_2 in the anaerobic treatments.

as an electron donor, and nitrous oxide was observed as an intermediate. Recalling that the ability to exploit ammonium as an electron donor is characteristic of *N. europaea*, this result further suggests that denitrification was indeed carried out by *N. europaea*.

When a higher cell density was used to inoculate the solution, slightly higher levels of dinitrogen production were observed. For example, when the cell density was 3.02 mg-wet cell/ml in the solution, the total conversion of NH_4^+–N to dinitrogen was 3.2%, which is similar to the 2.57% conversion efficiency observed in the 2.2 mg-wet cell/ml cell density. The small discrepancy may have been due to partial inactivation of the cell in the 2.2 mg-wet cell/ml cell density solution from the long incubation during ammonium oxidation.

Following incubation of five 50 ml bacterial solutions that ranged in pH from 6.0 to 8.0, studies revealed that pH value had a significant effect on the dinitrogen production (Fig. 4). Optimal pH for the production of dinitrogen was found to be 7.0–8.0. Values for the total conversion from NH_4^+–N to dinitrogen at pH values 7.0, 7.5 and 8.0 were found to be 1.74, 2.57 and 2.46%, respectively. Although the levels of dinitrogen production were similar at these pH values, no dinitrogen was detected at pH values below 7.0. This may be because nitrite spontaneously yields nitrosonium cations (NO^+) at lower pH values conditions via reactions of nitrous acid (HNO_2) with free protons [18]. Since nitrosonium cations are highly reactive with iron and copper, they readily form metal–nitrosyl complexes that prevent bacterial functions [19]. Therefore, it is likely that the lack of dinitrogen production at lower pH values may have been due to NO^+ production followed by inactivation of the bacterial enzymes.

To increase the dinitrogen production for 52 h solutions incubated were transferred into ammonium-free ATCC 1753 media. When 25 ml of ammonium-free fresh medium was inoculated with 25 ml of the 52 h incubated solution, conversion efficiency from NH_4^+–N to dinitrogen was found to be 6.74% (Fig. 5). This relatively higher dinitrogen production in the ammonium-free medium as compared to that in the

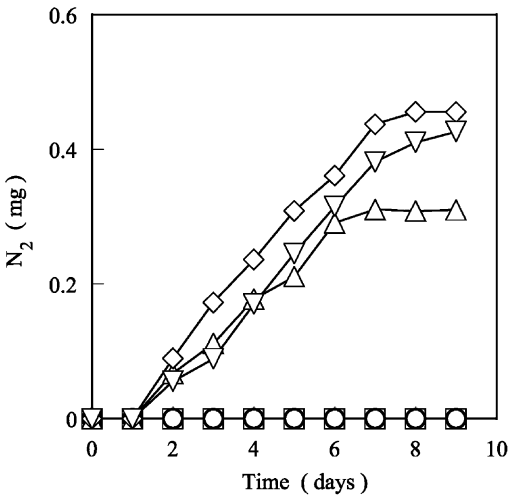

Fig. 4. Effects of pH on the production of dintrogen by *N.europaea* during denitrification. The serum bottles contained 50 ml of the solution described in the Fig. 1 caption. No production of dinitrogen was observed at acidic pH (6.0–6.5) but some was observed at pH 7.0–8.0. The symbols (□), (○), (△), (◇) and (▽) represent the dinitrogen production during denitrification at pH values of 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. All reactions were carried out in anaerobic conditions at 30 °C. Helium gas was used to replace O_2 in the anaerobic treatments.

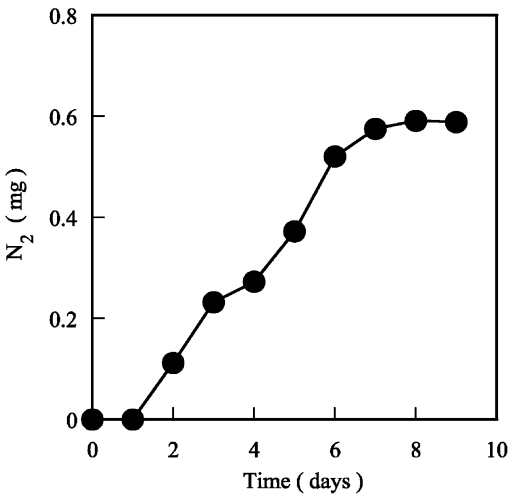

Fig. 5. Dinitrogen production following incubation of the air-supplied sample described in the Fig. 1 caption with ammonium-free ATCC 1573 medium. The serum bottles contained 25 ml of ammonium-free medium (pH 7.5) and 25 ml of air supplied solution (pH 7.5). Relatively higher dinitrogen production in the ammonium-free medium as compared to that in the original solution (solution without ammonium-free medium) was observed. The reaction was carried out at 30 °C in anaerobic condition, generated by the substitution of O_2 with helium gas.

original may be a form of inhibition caused by some unknown final product or may be due to an exhausted unbalanced culture. This indicates the necessity of fresh medium wherein bacteria can carry out denitrification after being transferred into serum bottles.

In the subsequent denitrification experiments, 50 ml of the above solution or 25 ml solution and 25 ml ammonium-free medium was transferred to 160 ml capacity serum bottles and deaerated by replacement with helium gas. Under these conditions, the maximum conversion of NH_4^+–N to dinitrogen was found to be 6.74%. However, when aerobically incubated cells were not transferred to new media for denitrification, the conversion of NH_4^+–N to dinitrogen was much lower (2.57%). This lower efficiency value likely reflects the necessity of fresh media for bacteria to carry out denitrification. The results of this study show that dinitrogen is produced by *Nitrosomonas* in a similar sequential pathway to that suggested for other denitrifiers: $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ [20,21].

As shown in Fig. 5, dinitrogen production stopped after 7 days of incubation. However, by the addition of fresh *N. europaea* cells at this time, dinitrogen production was resumed (data not shown) thus supporting the aforementioned idea that *N. europaea* cells were inactivated in these reaction conditions. Experiments geared towards obtaining prolonged dinitrogen production and complete conversion of ammonium to dinitrogen are currently in progress.

The results of this study suggest that dinitrogen conversion can be increased using high cell density. This clearly enables complete conversion of ammonium to nitrite within a short of period of time and most of the cells in the resulting solution remain active for denitrification. If a long time is required for the conversion, there is a high possibility of cell inactivation, consequently, dinitrogen production cannot be achieved.

4. Conclusion

Results of this study indicate that *N. europaea* cells are capable of simultaneous nitrification and denitrification at low partial O_2 pressure as well as in O_2-free conditions. If this process is carried out under controlled conditions, NH_4^+–N in wastewater can be converted to dinitrogen at a high conversion efficiency without employing large-scale separation of nitrification and denitrification.

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