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ORIGINAL RESEARCH PAPER

Biosynthesis of hydrazine from ammonium and hydroxylamine using an anaerobic ammonium oxidizing bacterium

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Abstract
Objectives To synthesize hydrazine (N_2H_4) from ammonium and hydroxylamine (NH_2OH) using an anaerobic ammonium oxidation (anammox) bacterium, *Candidatus Kuenenia stuttgartiensis*.
Results *K. stuttgartiensis* cells were anoxically cultivated with the addition of ammonium (2 mM) and NH_2OH (1–100 mM) at pH 6–10.5, and 4–65 °C to examine the favorable cultivation conditions for N_2H_4 production. The influence of NH_2OH concentration was more prominent than that of pH and temperature, and NH_2OH concentration higher than 1 mM deteriorated N_2H_4 yields significantly. The following conditions were found to be favorable for N_2H_4 production using *K. stuttgartiensis* cells: pH 9, 38 °C, and < 1 mM NH_2OH. In a continuous-feed system operated at these conditions, *K. stuttgartiensis* cells produced N_2H_4 with a maximum concentration of 0.65 mM, which is the highest N_2H_4 concentration previously reported in biological processes.
Conclusions Optimal cultivation conditions for *K. stuttgartiensis* for N_2H_4 production were successfully determined, and the present study is the first to document potential biological N_2H_4 production using anammox bacteria.

Keywords Anaerobic ammonium oxidation (anammox) · Anammox bacteria · Hydrazine production · Hydroxylamine

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Introduction

Hydrazine (N_2H_4) is a nitrogenous compound with a single N−N bond. This reactive molecule and its derivatives have a variety of industrial uses in, foaming agents, reducing agents, polymerization catalysts, precursors to pharmaceuticals and pesticides, and rocket fuels (Patil and Rattan 2014). N_2H_4 may be synthesized via many routes. Commercially, they are produced via chemical synthesis methods such as the Raschig process and ketazine process; however, these processes are known to have two disadvantages. First, large amounts of energy inputs are required during the heating process and second, inorganic salts are produced as byproducts (Schmidt 2000).

Anaerobic ammonium oxidation (anammox) is a microbial process in which NH_4^+ is oxidized to N_2 gas with NO_2^- reduction. This microbial process is only mediated by specific bacterial (i.e. anammox bacteria), which are affiliated with a monophyletic clade in the order *Brocadiales* in the phylum *Planctomycetes* (Kartal and Keltjens 2016). Notably, anammox bacteria synthesize N_2H_4 from NH_4^+ and NO or NH_2OH as an intermediate of the anammox process (Oshiki et al. 2016a), and biosynthesis of N_2H_4 has not been described for microbes other than anammox bacteria. Biosynthesis of N_2H_4 using anammox bacteria is an attractive strategy because the bacterial synthesis occurs at ambient temperature and without the production of inorganic salts a byproduct. For commercial applications of this strategy, a better understanding of the physiological characteristics of the anammox bacteria is essential, while the optimal cultivation condition of anammox bacteria to produce larger amounts of N_2H_4 has never been investigated (Oshiki et al. 2016b).

The purpose of the present study was to determine the optimal cultivation conditions of anammox bacteria for N_2H_4 production, and to examine the potential for N_2H_4 production under the optimal conditions. For this purpose, an anoxic batch incubation of an anammox bacterium, *Candidatus Kuenenia stuttgartiensis*, was performed under various pH levels, temperature conditions and NH_2OH concentrations. *K. stuttgartiensis* accumulated N_2H_4 from the supplied NH_2OH and NH_4^+, and the concentration and yield of the produced N_2H_4 were determined. Continuous-feed incubation of *K. stuttgartiensis* was subsequently performed in a membrane bioreactor (MBR), and N_2H_4 productivity was examined under the determined optimal cultivation conditions.

Materials and methods

Anammox biomass

Granular biomass (2–3 mm of diameter) of the anammox bacterium *Candidatus K. stuttgartiensis* was collected from an up-flow column reactor (980 mL). The column reactor has been operated at 37 °C under anoxic conditions for more than 2 years with a continuous supply of inorganic media containing NH_4^+ and NO_2^- (each 5 mM) at a nitrogen loading rate of 5 kg-N m^{-3} day^{-1} (Tsushima et al. 2007). The collected biomass was homogenized using a glass tissue grinder (AsOne, Osaka, Japan), and used for subsequent experiments. The dominance of *K. stuttgartiensis* in the biomass had previously been investigated by amplicon sequencing analysis of the 16S rRNA gene (Oshiki et al. 2018).

Batch incubation

Biomass was suspended at 1.7 mg-protein mL^{-1} in the anoxic inorganic media containing 2 mM $(NH_4)_2SO_4$, 1.2 mM $MgSO_4{\cdot}7H_2O$, 0.9 mM $CaCl_2{\cdot}2H_2O$, 0.2 mM KH_2PO_4, 5 mM $KHCO_3$, and 0.5 mL L^{-1} trace element solution I and II (van de Graaf et al. 1996). The suspension was dispensed into 15 mL serum glass vials (Nichiden-Rika glass, Tokyo, Japan) in an anaerobic chamber, in which the oxygen concentration was maintained at lower than 1 ppm (Oshiki et al. 2016a). After sealing with butyl rubber stoppers and aluminum caps, the headspace was replaced with He gas (> 99.99995%). Anoxic stock solution of NH_2OH was dispensed using a gas-tight syringe, and the vials were incubated for up to 36 h in the dark. Liquid samples were collected every 2 h to determine the NH_2OH and N_2H_4 concentrations. The yield of N_2H_4 from NH_2OH was calculated by dividing the maximum N_2H_4 concentrations by the decreased NH_2OH concentrations.

In order to determine the optimal cultivation conditions, pH, temperature, and NH_2OH concentration were varied across the pH ranges of 6–10.5, 4–65 °C, and 1–100 mM NH_2OH, respectively. The pH of the prepared media was determined using a pH meter D-51 (Horiba, Kyoto, Japan), and adjusted by adding Good's buffer at a final concentration of 25 mM. The buffers used were 2-morpholinoethanesulfonic acid, monohydrate (MES) for pH 6, 3-(N-morpholino) propanesulfonic acid (MOPS) for pH 7, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) for pH 8, tricine for pH 8.8, N-cyclohexyl-2-aminoethanesulfonic acid (CHES) for pH 9, and N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) for pH 10–10.5.

Continuous-feed incubation

Biomass was incubated in a 1-L MBR equipped with a hollow fiber membrane unit (300 polyethylene tubes;

pore size, 0.1 µm; tube diameter, 1 mm; length, 70 mm) (Oshiki et al. 2013) (Fig. 1). The above inorganic medium containing 15 mM NH_2OH was continuously supplied into the bioreactor at a flow rate of 1.23 mL min^{-1}. In addition to this continuous supply, NH_2OH solution (100 mM) was manually supplemented to increase the concentration to 0.6 mM when the NH_2OH concentration decreased below 0.1 mM. The liquid volume of the culture was adjusted to 1 L by using a peristaltic pump (EYELA, Tokyo, Japan) connected to a liquid level sensor WRX-01 (AsONE, Osaka, Japan). The pH of the culture was adjusted to 9 by adding 25 mM CHES, and the MBR was incubated at 38 °C. The bioreactor was continuously sparged internally with N_2/CO_2 gas (4:1, v/v) at a flow rate of 4 mL min^{-1} to maintain anoxic conditions. The culture medium was mixed using a magnetic stirrer at 80 rpm.

Chemical analysis

A portion of the culture collected during the above batch and continuous-feed incubation, was filtered through a 0.45-µm pore PVDF filter. NH_2OH concentrations were determined colorimetrically (Frear and Burrell 1955). Samples were mixed with 0.48% (w/v) trichloroacetic acid, 0.2% (w/v) 8-hydroxyquinoline, and 0.2 M Na_2CO_3, heated at 100 °C for 1 min, and the absorbance was measured at 705 nm. N_2H_4 concentrations were also determined colorimetrically (Watt and Chrisp 1952). Samples were mixed with 0.12 M 4-dimethylaminobenzaldehyde, and the absorbance was measured at 460 nm. We also tried to determine the N_2H_4 concentration fluorometrically using a rhodol levulinate (RL) probe, which has been developed as a N_2H_4-specific fluorescence probe (Tiensomjitr et al. 2018). We synthesized the RL probe by following literature procedure and characterized it by ^1H and ^{13}C NMR (JNM-ECP-400, JEOL, Tokyo, Japan). However, we found that the RL probe reacts with not only N_2H_4 but also NH_2OH. Therefore, we determined the N_2H_4 concentration using only the above-mentioned colorimetric method.

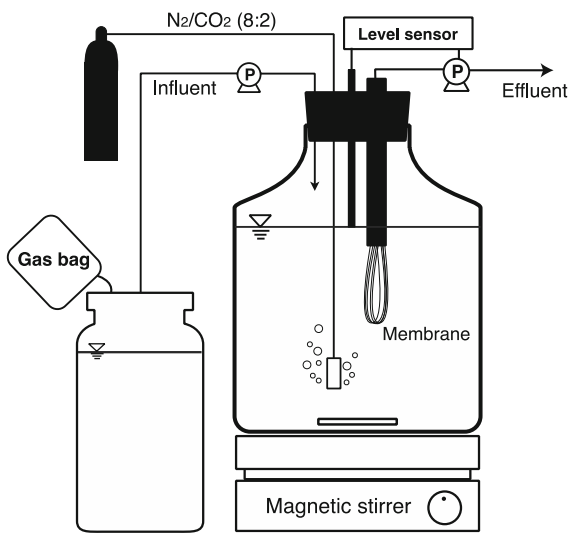

Fig. 1 Schematic drawing of membrane bioreactor. One-liter glass bottle equipped with a hollow fiber membrane (0.1 µm, polyethylene) unit was used as the culture vessel. The inside of the bioreactor was continuously purged with N_2/CO_2 mixed gas to prevent oxygen contamination. *P* peristaltic pump

Results and discussion

Determination of pH, temperature, and NH_2OH concentration for N_2H_4 production

The influence of pH levels, temperature conditions and NH_2OH concentrations on N_2H_4 production was investigated by batch incubation of *K. stuttgartiensis*. To examine the influence of pH, *K. stuttgartiensis* was incubated at 30 °C and the initial NH_2OH concentration was set to 2 mM. NH_2OH and N_2H_4 concentrations were determined every 2 h, and the incubation was continued until the increase of N_2H_4 concentration reached a plateau (within 36 h). As shown in Fig. 2a, the highest N_2H_4 yield (6.3%) was found at pH 9, and the N_2H_4 concentration increased to 0.06 mM after 18 h of incubation. N_2H_4 production was not detected when the incubation was carried out without *K. stuttgartiensis*. The batch incubations shown in Fig. 2 were not replicated and thus, the reproducibility was examined by incubating the vials at pH 9 and 30 °C with the addition of 2 mM NH_2OH in triplicate. The coefficient of variation of N_2H_4 yields was determined to be 4%. The influence of temperature was examined by incubating the biomass at different temperatures (4–65 °C). This incubation was performed at pH 9, with the addition of 2 mM NH_2OH. The highest N_2H_4 yield (2%) was found at 38 °C (Fig. 2b), and the maximum N_2H_4 concentration was 0.04 mM after 9 h of incubation. In addition to examining pH and temperature, the influence of the

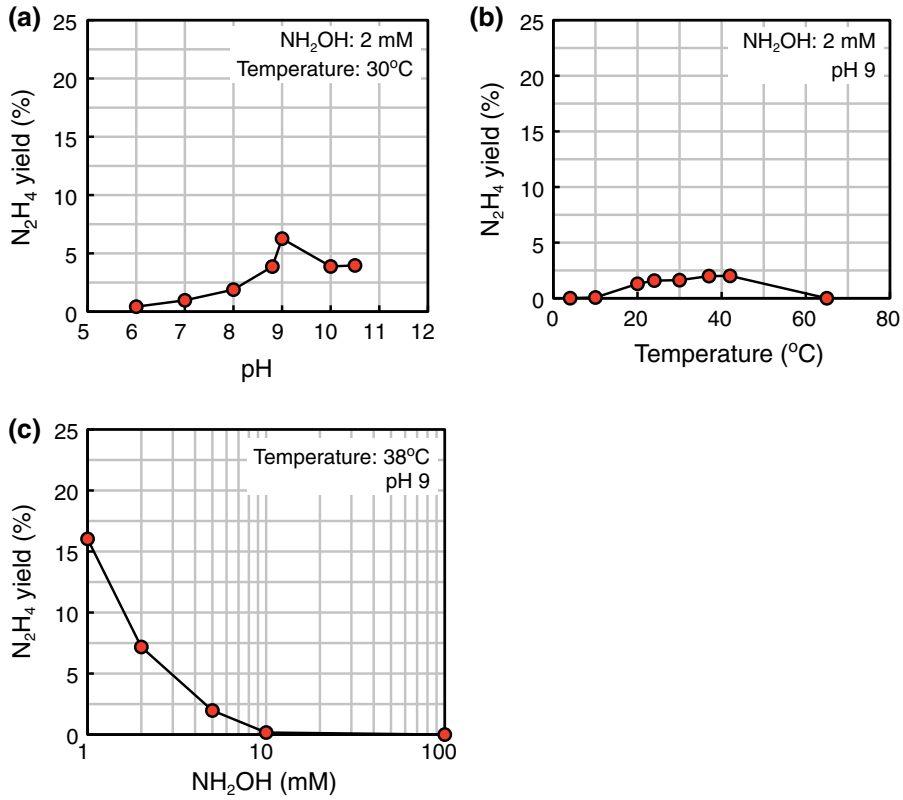

Fig. 2 Influence of pH **a**, temperature **b** and hydroxylamine (NH_2OH) concentrations **c** against N_2H_4 yield from NH_2OH. *K. stuttgartiensis* biomass was incubated in closed vials with addition of NH_4^+ and NH_2OH, and NH_2OH and N_2H_4 concentrations were determined via a time course. N_2H_4 yield was calculated by dividing the maximum N_2H_4 concentration by the decreased NH_2OH concentrations

NH_2OH concentration was also determined. The biomass was incubated at pH 9 and 38 °C, and initial NH_2OH concentrations were set at 1 to 100 mM. Notably, a lower NH_2OH concentration substantially increased the N_2H_4 yield (Fig. 2c). The highest N_2H_4 yield (16%) was found at 1 mM NH_2OH, and the maximum N_2H_4 concentration was 0.042 mM after 6 h of incubation. On the other hand, N_2H_4 production was absent when *K. stuttgartiensis* was incubated at 10 mM NH_2OH, indicating an inhibitory effect of NH_2OH at high concentrations. Based on the above findings, the optimal pH, temperature, and NH_2OH concentration for N_2H_4 production were set to be pH 9, 38 °C, and < 1 mM, respectively. The pH and temperature correspond to the upper limit of optimal pH and temperature ranges of *K. stuttgartiensis* (i.e. pH 6.5–9 and 25–37 °C, respectively) (Oshiki et al. 2016b).

Anammox bacteria, including *K. stuttgartiensis,* synthesize N_2H_4 using hydrazine synthase, which is subsequently oxidized to N_2 gas by hydrazine dehydrogenase (Hdh) under physiological conditions (Kartal and Keltjens 2016). Hdh (EC 1.7.2.8) is a N_2H_4-oxidizing octaheme protein that catalyzes the four-electron oxidation of N_2H_4 to N_2 gas (Shimamura et al. 2007). As N_2H_4 oxidation of Hdh is an undesirable reaction for N_2H_4 production, the activity of Hdh must be suppressed during N_2H_4 production. Notably, N_2H_4 oxidation of *K. stuttgartiensis* Hdh was inhibited by NH_2OH (7.9 μM of K_i values) (Maalcke et al. 2016); therefore, we examined the influence of NH_2OH on N_2H_4 production. As shown in Fig. 2c, NH_2OH can also inhibit N_2H_4 synthesis at high concentrations (i.e. > 1 mM); therefore, the NH_2OH concentration needs to be monitored and maintained below 1 mM during N_2H_4 production using *K. stuttgartiensis*.

N_2H_4 production during continuous-feed incubation

The potential for N_2H_4 production under the above optimal conditions was examined by continuously feeding NH_2OH into the *K. stuttgartiensis* culture. For this purpose, *K. stuttgartiensis* (3.4 mg-protein mL^{-1}) was cultivated at pH 9 and 38 °C in a 1-L MBR with a constant supply of 15 mM NH_2OH (1.23 mL min^{-1}).

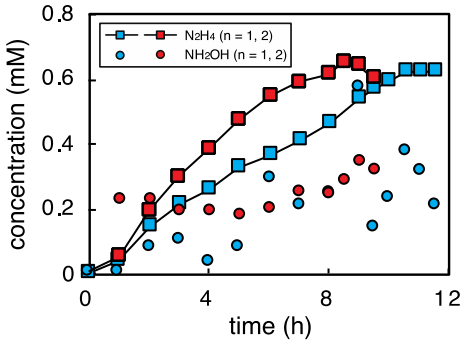

Fig. 3 N_2H_4 production during continuous-feed incubation *K. stuttgartiensis* (3.4 mg-protein mL^{-1}) was incubated at pH 9 and 38 °C in a 1-L membrane bioreactor with continuous supply of 15 mM NH_2OH (1.23 mL min^{-1}). The supplied NH_2OH was consumed by the biomass, and the concentration was maintained below 0.6 mM during the incubation

The supplied NH_2OH was consumed in the reactor continuously, and the NH_2OH concentration was maintained below 0.6 mM during 12 h of incubation (Fig. 3). This concentration range was lower than the inhibitory concentration of NH_2OH (i.e. > 1 mM) observed in the above batch incubation (Fig. 2c). As shown in Fig. 3, the N_2H_4 concentration increased up to 0.62 and 0.65 mM (n = 1 and 2, respectively) with production rates of 56 and 77 μM h^{-1}, respectively. We repeated the incubation in which the biomass and NH_2OH concentration decreased from 3.4 to 1.7 mg protein mL^{-1} and 15 to 10 mM, respectively. In this case, the maximum N_2H_4 concentration was 0.56 mM, and the N_2H_4 production rate was 74 μM h^{-1}. The maximum N_2H_4 concentration found during continuous-feed incubation (i.e. 0.65 mM) was an order of

Table 1 N_2H_4 production in annamox bacterial cultures

Incubation[a]	Bacterial species	Biomass concentration	pH	Temp.(°C)	NH_2OH (mM)[b]	N_2H_4 (mM)[c]	Yield (%)[d]	N_2H_4 productionrate (μM h^{-1})	References
Continuous	*K. stuttgartiensis*	3.4 mg-protein mL^{-1} (n = 1)	9	38	< 0.6	0.62	*n.a*	56	This study
		(n = 2)	9	38	< 0.4	0.65	*n.a*	77	
		1.7 mg-protein mL^{-1}	9	38	< 0.3	0.56	*n.a*	74	
Batch	*K. stuttgartiensis*	5 mg-C mL^{-1}	7.5–8	37	6.6	0.15[e]	2.3	68	van der Star et al. (2008)
	Brocadia sinica	< 1 mg-protein mL^{-1}	7.6	37	1.5	0.26	18	88	Oshiki et al. (2016a)
	Brocadia fulgida	< 5 mg-protein mL^{-1}	7–7.3	33	4	0.19	4.8	54	Kartal et al. (2008)
	Jettenia caeni	< 0.6 mg-protein mL^{-1}	7.8	37	3	0.12	4	40	Ali et al. (2015)
	Anammoxoglobus propionicus	< 5 mg-protein mL^{-1}	7–7.3	33	5	0.3	6	600	Kartal et al. (2007)
	n.a	*n.a*	7.5	30	3	0.5	16.7	42	van de Graaf et al. (1997)

n.a not applicable and not available

[a]Batch and continuous-feed incubation, respectively, [b]Initial NH_2OH concentration at the batch incubations and the highest NH_2OH concentration found during the continuous-feed incubations, [c]The maximum N_2H_4 concentration observed during the incubations, [d]Yield of N_2H_4 production from NH_2OH consumption, which was calculated by dividing the maximum N_2H_4 concentration by the consumed NH_2OH concentration, and [e]The data obtained from a 15-L reactor

magnitude higher than that observed in the batch incubations, and was the highest among those previously reported from anammox bacterial cultures (Table 1). As for the N_2H_4 production rates, *Candidatus Kuenenia*, *Brocadia,* and *Jettenia* showed similar production rates, while *Anammoxoglobus* showed a production rate an order of magnitude higher (i.e. 600 μM h^{-1}) (Table 1). N_2H_4 production using *Anammoxoglobus* and the investigation of underlying mechanisms allowing high N_2H_4 production require further study. An enrichment culture of *Anammoxoglobus* had been obtained in a bioreactor fed with propionate in addition to NH_4^+ and NO_2^- (Kartal et al. 2007), however, the enrichment culture of this anammox bacterium has not been described other than the original report. Therefore, more efforts are required to determine the cultivation conditions of *Anammoxoglobus*.

The increase in N_2H_4 concentration during the continuous-feed incubation was halted after 11 and 9 h of incubation (n = 1 and 2, respectively) (Fig. 3). NH_2OH concentration did not increase even after 11 and 9 h of incubation, indicating that *K. stuttgartiensis* still consumed the supplied NH_2OH. Therefore, our findings suggest that an increase in N_2H_4 oxidation activity resulted in the saturation of N_2H_4 production. Suppression of enzymatic activity and/or expression of Hdh is expected to contribute to the increase in the maximum N_2H_4 concentration; for example, nitric oxide has been recognized as another inhibitor of *K. stuttgartiensis* Hdh with the K_i value of 2.5 μM (Maalcke et al. 2016).

Conclusion

Here, we described favorable pH, temperature, and NH_2OH concentrations for biological N_2H_4 production using *K. stuttgartiensis* (i.e. pH 9, 38 °C and < 1.0 mM NH_2OH, respectively). Combined use of these conditions in continuous-feed incubation achieved 0.65 mM N_2H_4 concentration, which was the highest N_2H_4 concentration described in the biological process. N_2H_4 consumption by *K. stuttgartiensis* became prominent at the end of the continuous-feed incubation, which resulted in the saturation of N_2H_4 production. To enhance N_2H_4 productivity using anammox it is essential to screen for inhibitors that can specifically inhibit N_2H_4 oxidation of anammox bacteria.

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Author contributions All authors contributed to the study conception and design. Material preparation was performed by MO, IK, KO, and TI. Data collection and analysis were performed by MO and KY. The first draft of the manuscript was written by MO and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study does not include any studies with human participants or animals performed by any of the authors.

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