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Removal of a High Load of Ammonia by a Marine Bacterium, *Vibrio alginolyticus* in Biofilter

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Abstract A newly isolated heterotrophic marine bacterium, *Vibrio alginolyticus*, was used to remove a high load of ammonia gas under non-sterile condition. The cells were inoculated onto an inorganic packing material in a fixed-bed reactor (biofilter), and a high load of ammonia, in the range of ammonia gas concentration of 170 ppm to 880 ppm, was introduced continuously. Sucrose solution and 3% NaCl was supplied intermittently to supplement the carbon source and water to the biofilter. The average percentage of gas removed exceeded 85% for 107-day operation. The maximum removal capacity and the complete removal capacity were 19 g-N kg^{-1} dry packing material day^{-1} and 16 g-N kg^{-1} dry packing material day^{-1}, respectively, which were about three times greater than those obtained in nitrifying sludge inoculated onto the same packing material. On day 82, the enhanced pressure drop was restored to the normal one by NaOH treatment, and efficient removal characteristics were later observed. During this operation, the non-sterile condition had no significantly adverse effect on the removability of ammonia by *V. alginolyticus*.

Keywords: ammonia removal, biofilter, marine bacterium, *Vibrio alginolyticus*

INTRODUCTION

For the treatment of malodorous gases, physical and/or chemical methods have been commonly used. Although the efficiency of these methods is generally satisfactory, their maintenance and operation costs are high and thus, the alternative use of biological methods has attracted much attention. Ammonia gas is a notably volatile malodorous gas among malodorous gases. Among the biological methods for the removal of ammonia, the use of autotrophic nitrifying bacteria and a packed-bed reactor system called a biofilter has been reported to be an efficient method under a relatively low load of ammonia [1-12]. However, the utilization of nitrifying bacteria has several disadvantages as follows: (i) ammonia gas concentration exceeding 35 ppm and high load of ammonia are toxic to most nitrifying bacteria [4,5], (ii) nitrifying bacteria are adversely affected by shock loading and high organic loads [13], (iii) growth rates of autotrophic nitrifying bacteria are extremely low, and it sometimes takes one to two months to reach a steady state, (iv) contamination by heterotrophic bacteria often disturbs the system and (v) quantitative determination of nitrifying bacteria is time-consuming. To overcome the disadvantages posed by nitrifying bacteria, immobilization of cells and the use of heterotrophic bacteria are possible countermeasures [14-17]. A high concentration of ammonia of a few thousands ppm is emitted from fertilizer companies, compost facilities and livestock buildings and conventional nitrification method cannot be applied without dilution of the inlet gas. Furthermore, it is necessary to minimize the time to reach steady state and the fluctuation of the biofilter by the disturbance of the inlet concentration or the contaminants. We are interested in using marine bacteria because the high salinity environment is not optimal for the growth of most microorganisms and a halophilic environment may compensate the disadvantages of nitirifers.

In this study, we demonstrate the possibility of using a newly isolated marine bacterium, *Vibrio alginolyticus*, to remove a high load of ammonia. Since *V. alginolyticus* is a heterotrophic and halophilic bacterium, supply of a carbon source and NaCl is essential to maintain its activity. As a carbon source, sucrose was used. The ammonia removal capacity of the bacterium was investigated in a non-sterile halophilic medium for a period of 117 days where the effect of contamination was expected to be minimized.

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MATERIALS AND METHODS

Strain and Media Used

V. alginolyticus was isolated from seawater by enrichment culture after supplying a high ammonia concentration in Kanagawa Institute of Health Science, Kanagawa, Japan and identified by the National Collections of Industrial and Marine Bacteria, Japan, Co., Ltd. The strain was preserved in a heart infusion broth (HIB) (Difco Lab., Detroit, USA) containing 1% NaCl at -80°C.

The sucrose medium contains in one liter 30 g of sucrose, 15 g of KH_2PO_4, 36 mg of $MgSO_4 \cdot 7H_2O$, 1.3 mg of $CaCl_2 \cdot 2H_2O$, 0.7 mg of $ZnCl_2$, 1 mg of $FeSO_4 \cdot 7H_2O$ and 30 g of NaCl. The pH was adjusted to 8.0. This medium was used without sterilization. To determine the viable cell number of *V. alginolyticus*, heart infusion broth agar (HIBA) (pH 7.4) medium containing 1% agar and 3% NaCl was used. During ammonia gas supply experiments under non-sterile conditions, bacterial and fungal contaminants were detected using nutrient broth agar (NBA) medium containing 5 g of NA-yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 10 g of Polypepton (Nippon Seiyaku Co., Ltd., Osaka, Japan) and 15 g of agar per liter (pH 7), and potato dextrose agar (PDA) medium (Difco Lab.) (pH 6.2), respectively. The viable cell numbers of these microorganisms were measured after 1-7 days of incubation at 30°C and expressed as colony forming units (cfu).

Cultivation of *V. alginolyticus*

The cells of *V. alginolyticus* were grown in 5 mL HIB containing 3% NaCl in a test tube (16 mm diameter × 180 mm length) at 30°C, and shaken at 150 strokes per minute (spm) for 8 h. The cells were harvested by centrifugation at 8000 × *g* for 10 min and washed twice with 3% NaCl. A 3-mL aliquot of the cell suspension was inoculated into 300 mL of the sucrose medium in a shaken flask (500 mL nominal volume) which was sterilized beforehand at 120°C for 20 min. Ten g of steam-sterilized inorganic packing material was added and membrane-filtered $(NH_4)_2SO_4$ solution was also added to a final concentration of 2 g/L and shaken at 100 spm at 30°C for 24 h.

Biofilter Experiment

Biofilter experiments were conducted under non-sterile conditions at a temperature of 20-25°C. The laboratory-scale biofilter system is shown in Fig. 1. A glass column (50 mm inner diameter, 500 mm height, volume 0.98 L) was packed with an inorganic packing material, Fuyolite (no. 7, Fuyo Perlite Co., Ltd., Tokyo, Japan), to a height of 200 mm (volume 0.31 L), which was immobilized with cells of *V. alginolyticus* grown in a shaken flask for 24 h as described above. Ammonia gas in a cylinder (5% in N_2, Takachiho Chemical Co., Ltd., Tokyo, Japan) diluted with air was supplied into the glass column from the top downward in the flow rate range of 0.6-1.6 L/min. The sucrose medium was supplied using a peristaltic pump at 0.75 mL/min for 1 h four times a day in order to supplement water and a carbon source to the packing material and the cells. A total of 180 mL of fresh sucrose medium was supplied daily to the packing material and about 160 mL of the eluate was drained out from the bottom of the packed bed column. The eluate was used for the analysis of pH, sugar concentrations and cell numbers. Fuyolite as a packing material is obsidian sintered to form a foam aggregate which is mainly composed of 78.2% SiO_2 in 96.7% ash. Its average particle size is 4 mm and bulk density is 0.12 g cm^{-3}. The characteristics of this inorganic packing material in the biofilter used in the removal of ammonia and inoculated with nitrifying sludge were described in a previous paper [18]. Table 1 shows the experimental conditions.

Table 1. Experimental conditions of biofilter using *V. alginolyticus* for ammonia gas removal

Initial pH	8.0
Initial dry weight of Fuyolite (g)	34.7
Initial packed volume (L)	0.39
Initial packed height (cm)	20
Initial packed density (g-dry/L)	89
Flow rate (L/min)*	0.6-1.6
Space velocity (h^{-1})*	92-244
Inlet NH_3 concentration (ppm)*	170-880
NH_3 load (g-N kg^{-1} dry packing material day^{-1})*	5.3-19.6

* Minimum and maximum values

Analytical Methods

Ammonia concentrations at the inlet and the outlet of the gas line were measured using different types of gas-detection tubes (Gastec Co., Ltd., Tokyo), depending on the concentration. The measurement error using the tubes was ±5%. At periodic sampling times, the pH of the eluate from the biofilter was measured. Then, the eluate was centrifuged at 8000 × *g* for 10 min and the supernatant was filtered through a 0.2-μm membrane filter (DISMIC-25HP, Advantec Co., Ltd., Tokyo, Japan). Nitrite and nitrate concentrations of the filtrate were detected using Merckoquant test strips (Merck KgaA, Germany).

Glucose, fructose and sucrose concentrations in the eluate were determined by high-performance liquid chromatography (HPLC) (Shodex · NH2P-504E, Showa Denko Co., Ltd., Tokyo, Japan), using a mixture of ethanol and acetonitrile (3:1, v/v) as the eluent, at a flow rate of 1 mL/min at 40°C.

Ammonium ion concentration in the medium was measured by indophenol method. Amino acids were analyzed by thin-layer chromatography and an amino acid analyzer.

Intermittently, 10 g (wet) of Fuyolite was sampled from the biofilter and 90 mL of 1% sterile NaCl solution was added. The Fuyolite suspension was disrupted by homogenization at 5000 × *g* for 5 min and the measurement of pH, sugar concentrations and microbial counts were carried out using the same procedure described above. About 4 g of Fuyolite was dried at 105°C for 48 h and the water content was calculated.

Recover of Pressure Drop

On day 82 of the operation, the pressure drop of the biofilter reached about 220 mm H_2O/m, and the pressure drop under various flow rates was measured. Then,

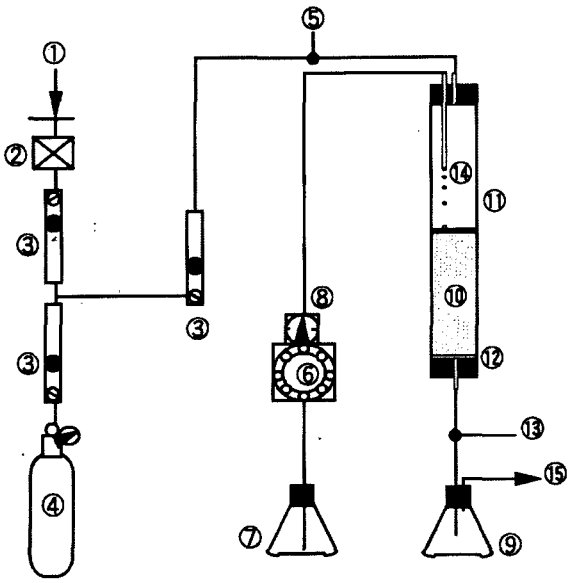

Fig. 1. Schematic diagram of the laboratory-scale biofilter for ammonia removal by the marine bacterium *V. alginolyticus* when Fuyolite was used as an inorganic packing material. (1) pressurized air; (2) regulator (1 kg/cm^2); (3) flowmeter; (4) 5% concentrated ammonia gas cylinder; (5) inlet gas sampling port; (6) peristaltic pump; (7) sucrose medium supply tank; (8) timer; (9) drain trap; (10) Fuyolite; (11) column; (12) saran net; (13) outlet gas sampling port; (14) sprinkler; (15) gas outlet.

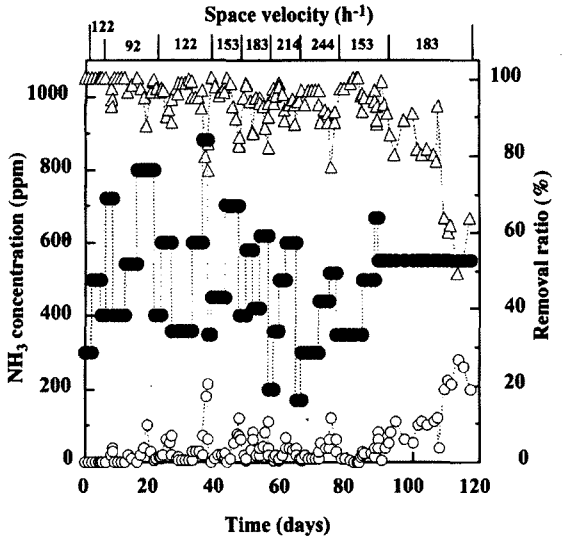

Fig. 2. Time course of inlet (●) and outlet (○) concentrations of ammonia, space velocity and removal ratio (△) to the Fuyolite biofilter inoculated with the marine bacterium *V. alginolyticus*. Gas supply and medium supply were conducted under non-sterile conditions.

the packing materials were removed from the column and suspended in 0.5 N NaOH for 5 min and were rinsed with distilled water until the pH became approximately 8. Then, the packing materials were refilled into the column, the air flow rates were changed for a short period and the pressure drop after NaOH treatment was measured. After the experiment was resumed, the periodical change in pressure drop was monitored at a constant space velocity (= gas flow rate/volume of packing material) of 183 h^{-1}

RESULTS

Biofilter Test

The inlet and outlet concentrations of ammonia gas, and change in space velocity in the Fuyolite biofilter are shown in Fig. 2. Ammonia concentration at the inlet was changed from 170 ppm to 880 ppm over a period of 117 days in the range of space velocities of 92 h^{-1} and 244 h^{-1}. During the experimental period, the inlet load of ammonia was varied from 5.6 g-N kg^{-1} dry packing material day^{-1} to 19.6 g-N kg^{-1} dry packing material day^{-1}. Over 10 days from the start of the experiment, the inlet ammonia gas concentration was increased from 300 ppm to 700 ppm at a constant space velocity of 122 h^{-1}, and ammonia was detected at the outlet. Therefore, the inlet concentration and the space velocity were decreased so that ammonia would not be detected at the outlet. Then, the inlet ammonia concentration was gradually increased. These procedures were repeated so as to maintain 100% removal and the inlet concentration was finally increased to 880 ppm. During the 117-day operation, an average percentage of removal of 85% was attained. When the load of ammonia exceeded about 10 g-N kg^{-1} dry packing material day^{-1}, ammonia was detected at the outlet. On day 82, recovery of the pressure drop was carried out. The ammonia supply was resumed at a constant inlet concentration of 350 ppm at a space velocity of 153 h^{-1} (load, 8.2 g-N kg^{-1} dry packing material day^{-1}) and increased to 670 ppm (load, 15.7 g-N kg^{-1} dry packing material day^{-1}) in 7 days and more than 85% of removal ratio was obtained. From 92 to 117 days, a constant inlet ammonia concentration of 550 ppm, and a constant space velocity of 183 h^{-1}, were fixed and on 107 days the concentration of sucrose in the intermittent supply was reduced to half of the previous one. Consequently, the removal ratio decreased significantly and the increase in outlet concentration of ammonia became obvious. This indicates that the concentration of sucrose in the feed was balanced to the high load of ammonia to maintain high removal ratio of ammonia.

Change in pH and Water Content

Fig. 3 shows the change of pH in the eluate and of the Fuyolite and the water content during 117 days of operation. No specific control of pH was conducted during the experimental period, but the pH values in the eluate and of the packing material were maintained

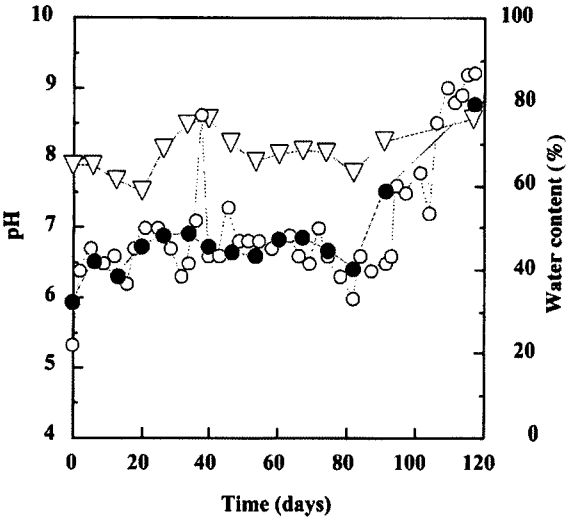

Fig. 3. Change in pH on the packing material, Fuyolite (●), in the eluate (○), and water content of the Fuyolite (▽). The supply of sucrose medium for moisture control was conducted under non-sterile conditions.

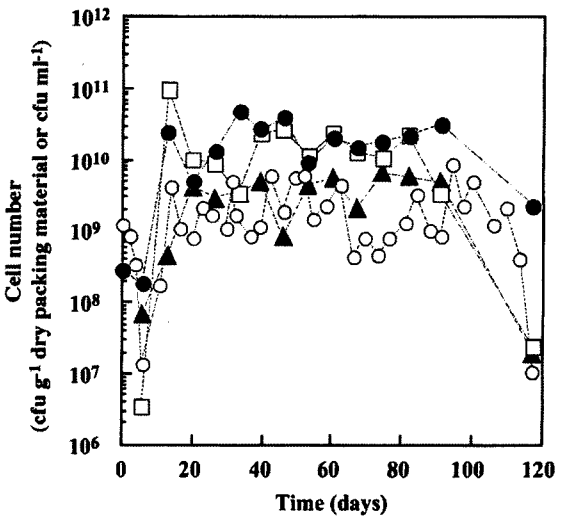

Fig. 4. Change in viable cell numbers of *V. alginolyticus* on the packing material (●) and in the eluate (○) and contaminants that appeared on NBA medium (□) and PDA medium (▲) for the Fuyolite biofilter.

in the range of 5.3 to 9.2 and 6.0 to 8.8, respectively. When the removal rate deteriorated, especially after 107 days when the sucrose concentration was reduced, the value of pH increased mainly due to the accumulation of NH_4^+ ions. The pH value tended to increase when sucrose was exhausted. The water content of the packing material was kept at approximately 70% during the experimental period. The water loss of an inorganic packing material like the Fuyolite by evaporation is serious especially when space velocity is high [18], and the intermittent supply of the sucrose medium in this experiment was appropriate for maintaining the water content at 70% for optimal microbial activity.

Microbial Count

Contamination by foreign microbes is inevitable in a non-sterile system. The microbial count of *V. alginolyticus* and the number of colonies that appeared on NBA and PDA media from the eluate and the Fuyolite packing material are shown in Fig. 4. The initial viable cell number of *V. alginolyticus* on the packing material was 2.8×10^8 cfu/g dry packing material and 2 weeks later, the maximum viable cell number of *V. alginolyticus* reached 2×10^{10} cfu/g dry packing material and more than 1×10^{10} cfu/g dry packing material was maintained. The average microbial numbers of *V. alginolyticus* in the eluate and on the packing material were 3.1×10^9 cfu/mL and 2.2×10^{10} cfu/g dry packing material. The average microbial counts on PDA and NBA media were 1.4×10^9 and 4.8×10^9 cfu/g dry packing material, respectively, which are 1/5-1/15th of the values for *V. alginolyticus* grown on the Fuyolite. Therefore, the contribution of these contaminants to ammonia removal is assumed to be negligible, although the nitrogen uptake rate of these contaminants was not measured. On day 117, a significant decrease in viable cell numbers was observed mainly because the reduced supply of sucrose reflected in decrease in active cell number and in the increase in pH values (Fig. 3).

The high concentration of the marine bacterium on the Fuyolite at the high load of ammonia and at the sucrose supply indicates that the inoculated bacterium was stably maintained during operation and no takeover by contaminant microorganisms occurred. This is advantageous for heterotrophic bacteria because autotrophic bacteria were easily dominated by heterotrophic bacteria under excess nutrient conditions.

Change in Sugar Concentrations

Changes in sucrose concentration in supply medium and fructose, glucose and sucrose concentrations in the eluate were followed (data not shown). Peaks of sucrose concentration in the eluate were observed at each supply time of sucrose. The detection of fructose and glucose concentrations was due to the conversion of sucrose to glucose and fructose by *V. alginolyticus* and probably by contaminants. No sugar was detected from the packing material, indicating that the activity of the immobilized cells on the packing material was sugar-limited. The average consumption rate of three sugars are listed in Table 2. *V. alginolyticus* showed a high consumption rate for sucrose and a similar consumption rates of glucose and fructose. After 107 days, the reduction of inlet sucrose concentration severely affected the activity of microorganisms on the packing material, and thus the outlet concentrations of sucrose increased.

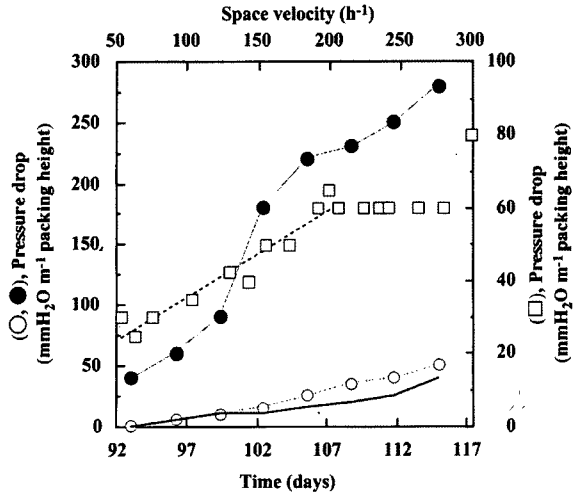

Fig. 5. Changes in pressure drop of Fuyolite biofilter before NaOH treatment (●) and after NaOH treatment on day 82 (○) at various space velocities and change in pressure drop of the fresh Fuyolite biofilter (—). Time course of pressure drops (□) from days 92 to 117 at space velocity of 183 h^{-1} after NaOH treatment of the Fuyolite on day 82.

Table 2. Experimental results of biofilter using *V. alginolyticus* for ammonia gas removal

Maximum removal capacity (g-N kg^{-1} dry packing material day^{-1})	19
Complete removal capacity (g-N kg^{-1} dry packing material day^{-1})	16
Specific removal rate (g-N $cell^{-1} h^{-1}$)	3.1×10^{-14}
pH variation of packing material	6.0-8.8
Water content range (%)	58-76
Average cell number of *V. alginolyticus* (cfu/g dry packing material)	2.2×10^{10}
Average cell number of contaminated bacteria (cfu/g dry packing material)	4.8×10^{9}
Average cell number of contaminated fungi (cfu/g dry packing material)	1.4×10^{9}
Average consumption rate of sucrose (g kg^{-1} dry packing material day^{-1})	157
Average consumption rate of glucose (g kg^{-1} dry packing material day^{-1})	37
Average consumption rate of fructose (g kg^{-1} dry packing material day^{-1})	44

Recovery of Pressure Drop

When heterotrophic bacteria are immobilized on packing materials, the cell accumulation rate on the packing material is higher due to their high growth rate and accumulation of products such as polysaccharides. This causes the enhancement of pressure drop. During the experimental period, a gradual increase in pressure drop was observed and on day 82, the value reached approximately 220 mmH_2O/m at a space velocity of 153 h^{-1}. Then, the recovery of pressure drop was conducted. Fig. 5 shows the change in the pressure drop of the biofilter on day 82 when the space velocity was varied from 61 to 276 h^{-1}. After the treatment of the packing material with 0.5N NaOH solution, the change in the pressure drop of the packing material was significantly reduced in the same space velocity range, and the value was almost the same as that of the fresh packing material. This indicates that the cells or the accumulated substances on the packing materials which are responsible for increase in pressure drop are removed by the NaOH treatment. However, this treatment did not exert any adverse effect on the ammonia removal ability, as shown in Fig. 2. Then, the change in pressure drop of the recovered packing material was monitored from day 92 to day 117. A constant increase in the pressure drop, 2.4 mm H_2O m^{-1} day^{-1}, was observed. This value can be used to estimate the recovery time.

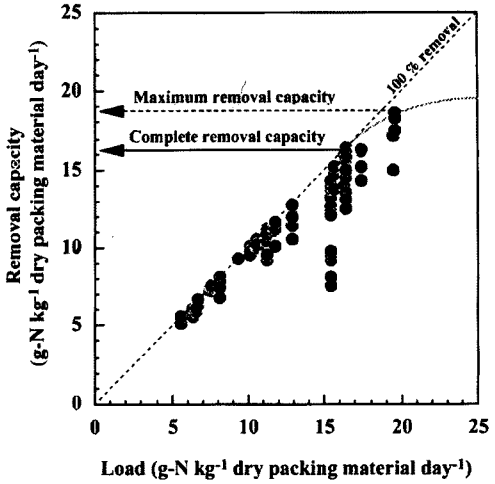

Fig. 6. Relationship between ammonia load and removal capacity in the biofilter with Fuyolite as the packing material inoculated with *V. alginolyticus*.

Estimation of Removal Capacity

The relationship between ammonia load and removal capacity is shown in Fig. 6. The complete removal capacity, which means 100% removal with respect to the inlet load, was 16 g-N kg^{-1} dry packing material day^{-1}. The maximum removal capacity was estimated to be 19 g-N kg^{-1} dry packing material day^{-1} by extrapolating the curve to the plateau value. We determined the complete removal capacity and the maximum removal capacity of ammonia using long-term acclimated nitrifying sludge immobilized on the same Fuyolite biofilter to be 4.7 g-N kg^{-1} dry packing material day^{-1} and 6.0 g-N kg^{-1} dry packing material day^{-1}, respectively [18]. Therefore, those capacities for *V. alginolyticus* are three times larger than those for the nitrifying sludge. The complete removal load of ammonia by other biofilters inoculated with nitrifying bacteria was reported to be in the range of 0.2-3 g-N kg^{-1} dry packing material day^{-1} [2,7,9-11].

Table 3. Nitrogen balance in ammonia removal by *V. alginolyticus* where the data from reference [23] are used

(unit. g-N/L)

Removed NH_3-N	Cellular N	NH_4^+-N in cell free culture	Alanine
8.55	0.27	5.5	3.1

DISCUSSION

By using a heterotrophic marine bacterium, a high load of ammonia from the start of cultivation could be applied. For nitrifying bacteria, direct introduction of 300 ppm of ammonia from the start of experiments had an severe inhibitory effect on growth and at 880 ppm, they could not carry out nitrification [10,11]. In this respect, *V. alginolyticus* will be applicable for the removal of relatively high loads of ammonia.

The specific ammonia removal rate was calculated from the average viable cell number of 2.2×10^{10} cfu/g dry packing material and the complete removal capacity, 16 g-N kg^{-1} dry packing material day^{-1} as 3.1×10^{-14} g-N $cell^{-1}\,h^{-1}$. Based on a kinetic study, ammonia oxidation by autotrophic bacteria has been reported to be in the range of 6×10^{-14} to 4×10^{-13} g-N $cell^{-1}\,h^{-1}$ [19-21], indicating that the specific nitrification rate of autotrophs is 2-10 times higher than that of heterotrophs. However, the cell concentration of heterotrophs can be $10\text{-}10^2$ times higher than that of autotrophs [19-21], mainly due to their high-energy consumption efficiency, and this can compensate for the ammonia removal capacity of heterotrophs. Since the total removability of ammonia in the system is dependent on the viable cell number and the specific ammonia removal rate, the higher capacity of the marine bacterium to remove ammonia lies in the maintenance of a high cell concentration. The immobilization of autotrophic bacteria was carried out to increase the population density of autotrophs, but the immobilization of the autotrophic bacteria resulted in a maximum ammonia load of 0.7-1.3 g-N L^{-1} day^{-1} [16,22], which was about 100 times lower than the value for *V. alginolyticus*.

The pH value can be maintained at 6-8 without particular control at the allowable space velocities, which is also advantageous for the practical use of this marine bacterium because nitrifying bacteria produce nitrite and nitrate via nitrification of ammonia and they cause the deterioration of the activity of the nitrifiers. Another advantage of using this marine bacterium is that bacterial or fungal contaminants can exist in the system but the effects of these contaminants are minimized mainly by the halophilic environment. Recently, some bacteria with heterotrophic nitrification ability were identified [14,15,17], but their nitrification rate is $1/100^{th}$ of that of autotrophic nitrifiers and the influence of contaminants on nitrification is significant. The immobilization of heterotrophic nitrifiers increased the ammonia removal rate only to 1.22 g-N kg^{-1} dry packing material day^{-1} [15].

At present, the mechanism of ammonia removal by *V. alginolyticus* is not fully understood, but the analytical data is shown. Since neither nitrite nor nitrate is produced by this marine bacterium, nitrification does not occur. Nitrogen balance and metabolite analyses based on pure culture of *V. alginolyticus* [23] is shown in Table 3. Ammonia gas was supplied to a vessel containing the cells of *V. alginolyticus* and the cells were grown for 6 days. Then, the nitrogen content of the cells and the composition of the cell free culture were determined. During 6 days operation, the total amount of removed nitrogen was 8.55 g-N/L and the cellular nitrogen was 0.27 g-N/L, and the cell-free culture contained 5.5 g-N of NH_4^+/L and 3.1 g-N of alanine /L, indicating the input and output of nitrogen was well balanced. This indicates that the uptake of ammonia by the cells, the reaction of ammonium ions with metabolites and conversion of ammonia to amino acids are the main reactions involved.

About 36% of removed nitrogen was converted to alanine by *V. alginolyticus*. The details of NH_4^+ compounds are not analyzed, but *V. alginolyticus* was reported to produce acidic products extracellularly like pyruvic acid and uronic acid that can neutralize ammonium ions [24]. In the biofilter experiment, the similar metabolism by *V. alginolyticus* may have occurred in spite of the existence of contaminated microorganisms. The contaminated microorganisms were supposed to utilize alanine or ammonium ions because only small amount of alanine or ammonium ions were detected in the eluate from the biofilter. This coexistence of the marine bacterium and the contaminants may lead to a stable removal of high load of ammonia gas in the biofilter.

CONCLUSION

A heterotrophic marine bacterium was firstly used to remove a high load of ammonia gas in a biofilter where nitrifying bacteria cannot be applicable. From the start of experiment, ammonia gas was removed efficiently without acclimation period of the bacterium to ammonia gas. High removability was maintained even under the non-aseptic condition. The accumulated cells on the inorganic packing material after a long operation were detached by NaOH treatment. As the maintenance of the heterotrophic marine bacterium is easier than that of autotrophic bacteria, the marine bacterium can give an alternative method to treat high concentration of ammonia gas.

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