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ORIGINAL PAPER

Ammonia removal from livestock wastewater by ammonia-assimilating microorganisms immobilized in polyvinyl alcohol

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Abstract We isolated ammonia-assimilating microorganisms from the livestock manure treatment systems and evaluated their ammonia-assimilating ability. Many isolates utilized ammonia at high rates when they were purely cultivated in a nitrogen-limited medium to which sterilized lagoon extract had been added. Some isolates that were immobilized in polyvinyl alcohol (PVA) utilized ammonia present in the media containing viable lagoon microorganisms. Staining with 4′,6′-diamidino-2-phenylindole (DAPI) indicated that the immobilized high ammonia-assimilating isolates grew dominantly within the PVA beads. High ammonia-assimilating isolates in the mixed culture containing viable lagoon microorganisms were identified as *Pseudomonas* spp. and member of *Rhizobiaceae* species by partial sequencing of the 16S ribosomal DNA.

Keywords Ammonia · Compost · Lagoon · Manure · PVA immobilization

Introduction

Ammonia is one of the offensive odorous materials which are generated from animal manure and its treatment processes, and it causes particulate formation [9]. Ammonia is utilized by various microbial species as a nitrogen source and is the most important nitrogenous compound in the nitrogen cycle in the livestock environment [4, 8, 12]. Organic nitrogenous compounds are degraded to ammonia in livestock waste treatment processes, and the generated ammonia is directed into the nitrogen cycle through two different biological processes. One process is the degradation of ammonia to nitrogen gas by oxidation and reduction, and the other is the assimilation of ammonia into microbial nitrogen compounds.

Nitrification is generally considered to be the major biological process undergone by ammonia during manure treatment. The first step is the oxidation of ammonia to nitrite by ammonia-oxidizing microorganisms, and the second step involves the oxidation of nitrite to nitrate by nitrite-oxidizing microorganisms. Subsequently, nitrate is reduced to nitrite, nitric oxide, nitrous oxide, and nitrogen gas. On the other hand, many species of heterotrophic and autotrophic microorganisms assimilate ammonia via glutamine synthesis [10, 14]. Although some microbial species are known to assimilate nitrate, they assimilate ammonia only after nitrate is reduced to ammonia by nitrate reductase [10]. Therefore, direct assimilation of ammonia by microorganisms may be more efficient than other biological processes such as the biomass production.

We have surveyed the distribution of ammonia-assimilating microorganisms in livestock manure treatment systems and isolated high ammonia-assimilating microorganisms [15–17]. There is a possibility that these microorganisms may not successfully compete with the natural inhabitants and may not be efficient in removing ammonia from the treatment systems. Recently, immobilized microbial cells have frequently been applied for bioremediation and biosynthetic

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processes [3, 5, 18]. The immobilized cells are easily prepared and are more stable than free-living cells over a long period. In particular, polyvinyl alcohol (PVA) is widely used for wastewater treatment processes. PVA gels are easily prepared from aqueous PVA solutions and have the characteristics of the high porosity and stability [7]. Although application of ammonia-assimilating microorganisms with PVA-immobilization is considered to contribute for ammonia removal from wastewater, the immobilized cells concerning ammonia assimilation had not yet been clarified.

In the present paper, we investigated the isolation of ammonia-assimilating microorganisms from livestock manure treatment systems and evaluated their ammonia-assimilating ability in the PVA-immobilized form in a mixed culture containing viable lagoon microorganisms.

Materials and methods

Culture medium

The nitrogen-limited medium that was used to isolate ammonia-assimilating microorganisms was composed of 5.0 g of glucose, 0.25 g of NH_4Cl, 0.1 g of $Fe(NH_4)_2H(C_6H_5O_7)_2$, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $MnCl_2{\cdot}4H_2O$, 3.2 g of K_2HPO_4 and 1.0 g of KH_2PO_4 in 1 l of distilled water. For the plate medium, 1.5% of gellan gum was added to the nitrogen-limited medium. Lagoon samples were taken from the surface of a lagoon basin that was used for the treatment of paddock of dairy cattle in Kawatabi farm of the Field Science Center, Graduate School of Agricultural Science, Tohoku University. Lagoon-extract medium was used to measure ammonia assimilation of the isolates. The medium was prepared by mixing lagoon-extracted solution and the nitrogen-limited medium (1:1). The lagoon-extracted solution was prepared in the following manner: lagoon samples were filtered through a double-folded gauze and centrifuged at 1,000*g* for 10 min. The pH of the supernatant was adjusted to 7.5 prior to use. The number of total aerobes in the nonsterilized lagoon-extract medium was approximately 10^7 cfu/ml. Ammonia concentrations of the sterilized lagoon-extract medium (pure culture) and nonsterilized lagoon-extract medium (mixed culture containing viable microbial flora) were 40.1 and 48.2 mg/l, respectively. PY agar that was used for maintaining isolated strains consisted of 0.5 g of tryptone peptone (BD, NJ, USA), 0.5 g yeast extract (BD), and 15 g of agar/l of water.

Isolation of ammonia-assimilating microorganisms

Ammonia-assimilating isolates, which were isolated from a lagoon used for treatment of wastewater from a paddock of dairy cattle by Sasaki et al. [15], were used in this study. Among these, very particularly high ammonia-assimilating isolates functioning at 20 and 25°C in pure cultures were selected for use.

Ammonia-assimilating isolates were also isolated from the compost samples. Samples were collected from a semi-closed type cubic-shaped composter. The raw materials of composting were a mixture of beef cattle waste and bark, used as the bedding material of cattle pens, and the mature compost. Compost samples were obtained from the initial stage of the composting phase and the last stages of the active composting phase and the composting phase. Ammonia-assimilating microorganisms were isolated by tenfold dilution steps using a nitrogen-limited medium. The nitrogen-limited medium was incubated at 37°C for 72 h under aerobic conditions. Each isolated bacterial colony was obtained as a pure culture by culturing twice in PY agar using the streak-plate method.

Preparation of PVA-immobilized cells

Preparation of PVA-immobilized cells was carried out accordingly to the method described by Chen et al. [2]. Three grams of PVA (Kurarey, Okayama, Japan) was dissolved in 20 ml deionized water and autoclaved at 121°C for 25 min. The polymer solution was cooled at 45°C and mixed with a preincubated cell culture (1:1). The mixture of PVA and the cell culture solution was dropped into saturated boric acid at 40°C and was solidified at room temperature for more than 20 h. Spherical beads of PVA-immobilized cells were obtained, and these were washed with tap water prior to use.

The ability of isolates to assimilate ammonia

Isolates were preincubated for 72 h in Trypto-soy broth (Nissui, Tokyo, Japan) at the same temperature at which they were isolated. Cells were harvested using centrifugation (15,000*g*, 15 min), washed twice with saline solution, and resuspended. The bacterial suspension was inoculated into the sterilized lagoon-extract medium in a proportion of 1:100 and was incubated for 72 h on the shaker (100 rpm) at the same temperature at which it was isolated.

For the culturing PVA-immobilized cells, 1 g-immobilized cells were inoculated into 25 ml of pure culture or mixed culture, and the culture was then incubated for 72 h on the shaker (100 rpm) at the same temperature.

The ammonia assimilation ability and nitrate and nitrite removal were determined by measuring the disappearance of ammonia, nitrate, and nitrite from the culture medium, respectively. Ammonia was measured by the indophenol method using ammonia test (Wako Pure Chemical, Osaka, Japan), and nitrite was measured by the diazotization method using NitriVer 3 Nitrite Reagent (Hach, CO, USA).

4′,6′-diamidino-2-phenylindole (DAPI) staining of the immobilized cells

The immobilized cells that showed high ammonia-assimilating ability (L2015) and low ammonia-assimilating ability (L2511) were visualized by DAPI staining. Prior to and after incubation, the PVA beads were cut with a sterilized cutter and were then stained with DAPI (1.5 μg/ml). The surfaces of the sliced samples were visualized with an epifluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan).

Identification of high ammonia-assimilating isolates

Isolates that showed particularly high ammonia assimilation in the PVA-immobilized culture were identified by the partial sequencing of the 16S ribosomal DNA (rDNA). The nucleotide sequences of the primers were as follows: primer 1 was 5′-CCTACGGGAGGCAGC AG-3′ and primer 2 was 5′-ATTACCGCGGCTGCT GG-3′ [11]. The optimized polymerase chain reaction (PCR) mixture contained 10× PCR amplification buffer, 15 mM $MgCl_2$, 2 mM dNTP, 25 μM each primer, and 1.25 U *Taq* DNA polymerase in a PCR reaction mixture having a total volume of 50 μl. Each bacterial colony was picked with a sterile toothpick and directly transferred to the PCR tube for use as the DNA templates. The thermal cycle program, run on a Model TP240 (Takara, Kyoto, Japan), consisted of cell lysis at 95°C for 15 min, denaturation at 94°C for 30 s, primer annealing at 57.5°C for 20 s, and extension at 72°C for 30 s. Amplification was repeated for 35 cycles and finally, extension was carried out at 72°C for 10 min. The amplified PCR products were purified with QIAquick PCR Purification Kit (Qiagen, CA, USA). The purified DNA was used as the template in the cycle sequencing reaction with BigDye Terminator Premix (Applied Biosystems, CA, USA) and purified with DyeEX Spin Columns (Qiagen). For cycle sequencing, 0.8 μM primers were used. The products of the sequencing reaction were analyzed with a model ABI 310 autosequencer (Applied Biosystems). The nucleotide sequences of selected isolates were compared with those of microorganisms deposited in GenBank.

Results

Ammonia-assimilating ability

Figure 1 shows the ammonia consumption of isolates from the lagoon in the lagoon-extract medium incubated at 20 and 25°C for 72 h. All isolates did not produce nitrite and those were confirmed as not being ammonia-oxidizing microorganisms. We have already isolated a total of 34 strains from the lagoon [15]. Nine of them were used in this study. In particular, L2013 and L2515 and L2516 that were isolated at 20 and 25°C, respectively, showed approximately 99% ammonia consumption by free cells of a pure culture. The ammonia-assimilating ability of the isolates was determined in the PVA-immobilized cells in the pure culture. L2013, L2014, and L2015 isolated at 20°C showed high ammonia consumption 42.2, 50.9, and 56.7%, respectively. Further, L2516 isolated at 25°C showed 25.6% ammonia consumption. The ammonia-assimilating ability of the isolates was also determined in the PVA-immobilized cells in the mixed culture containing microbial flora of the lagoon. Among the isolates, L2015 showed the highest ammonia consumption of 35.3%.

Figure 2 shows ammonia consumption of isolates from the composting processes during incubation at 37°C for 72 h. In particular, C10 and C24 isolated from the initial stage of composting and II14 from the last stage of the composting phase showed approximately 99% ammonia consumption by free cells of a pure culture. In the PVA-immobilized cells of the pure culture, they showed significant ammonia consumption in the range of 20.1–60.0%. Even in the mixed culture-containing lagoon isolates with PVA-immobilized cells, C24 and II14 showed 62.4 and 58.9% ammonia consumption, respectively.

Visualization of the immobilized cells

L2015 cells that showed high ammonia consumption of 35.3% in the mixed culture with PVA-immobilized cells were visualized with DAPI staining under the pure culture condition (Fig. 3a, b). L2511 cells that showed low ammonia consumption of 4.2% were also observed as low ammonia-consumption control (Fig. 3c, d). In both L2015 and L2511, the surface area of PVA gels showed a low density prior to incubation (Fig. 3a, c). After incubation, the surface area of L2015 in the PVA gel showed a markedly high density; however, that of L2511 did not show similar change in density at the beginning of incubation (Fig. 3b, d).

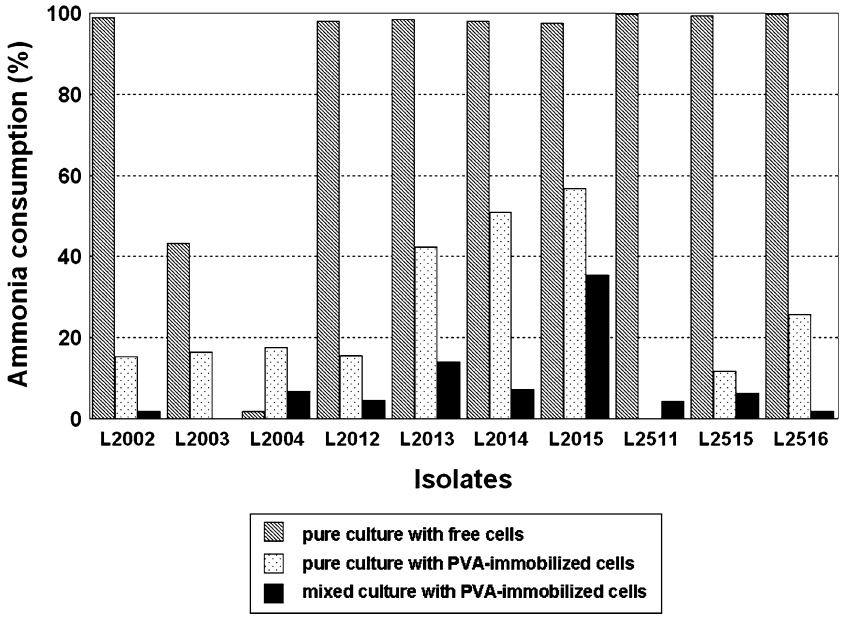

Fig. 1 Ammonia consumption of isolates from a lagoon incubated in a pure culture with free cells, in a pure culture with PVA-immobilized cells, and in a mixed culture with PVA-immobilized cells

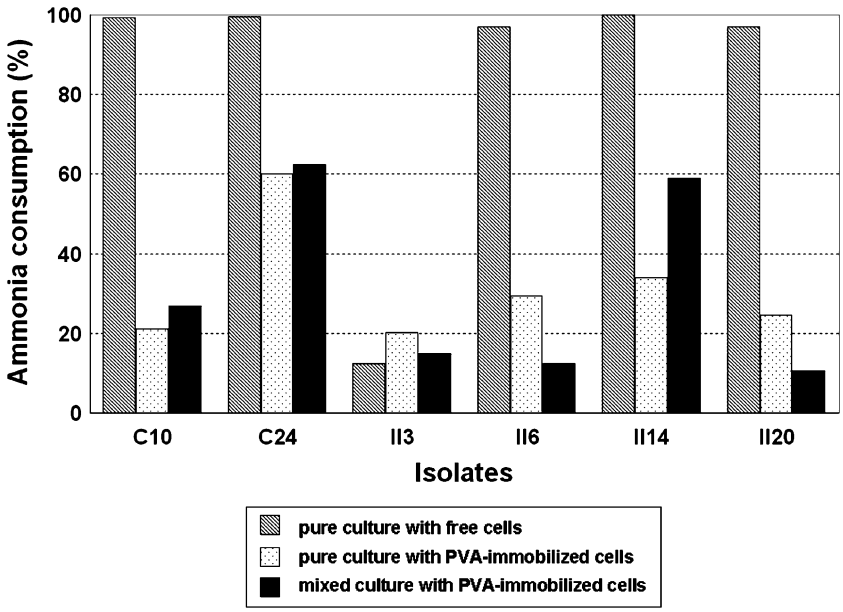

Fig. 2 Ammonia consumption of isolates from the compost sample incubated in a pure culture with free cells, in a pure culture with PVA-immobilized cells, and in a mixed culture with PVA-immobilized cells

Identification of high ammonia-assimilating isolates

L2015, C24, and II14 that showed high ammonia consumption in the mixed culture of the lagoon with PVA-immobilized cells were identified by partial sequence of the 16S ribosomal DNA. L2015 showed 100% similarity to *Pseudomonas* spp. (*P. coronafaciens* Z76660, *P. chlororaphis* Z76657, *P. fluorescens* AJ851196, *P. putida* D86003, *P. syringae* AY574914). The sequenced genes of both C24 and II14 were completely identical. Both showed 100% similarity to microorganisms belonging to *Rhizobiaceae*, e.g., *Rhizobium* sp. (AY776227), *Sinorhizobium* sp. (AY776197) and *Agrobacterium* sp. (AY776194).

Discussion

This is the first time to examine the microorganisms, which posse high ammonia-assimilating ability, with PVA immobilization in complex microbial conditions. Ammonia assimilation by heterotrophic microorganisms is important for prevention of ammonia volatilization from livestock manure treatment systems. Tanaka et al. [19] reported that the inoculation of high ammonia-assimilating filamentous fungi reduced ammonia volatilization from the composting processes, and they noted that the accumulated microbial protein in the composts was effective as a fertilizer. Potter et al. [13] reported that poly γ-D-glutamic acid (PGA)-producing

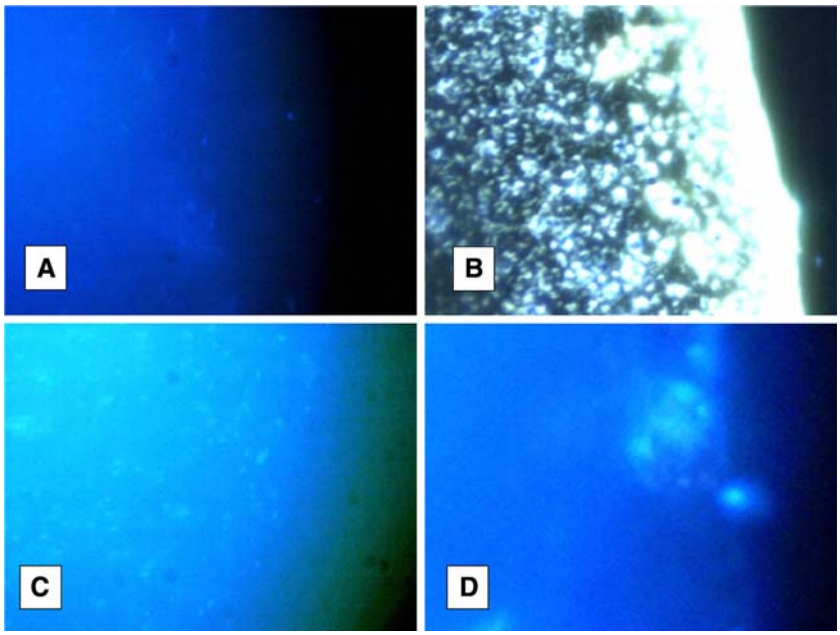

Fig. 3 DAPI staining of PVA-immobilized cells. **a** L2015 prior to incubation, **b** L2015 after incubation for 72 h in the pure culture, **c** L2511 prior to incubation, and **d** L2511 after incubation for 72 h in the pure culture

Bacillus assimilated 750 mg/l ammonia and produced 5 g/l PGA in liquid manure. Thus, control of ammonia volatilization is important for not only preserving the livestock environment but also for retaining of nitrogen compounds in the products.

All the isolates that grew well in the nitrogen-limited medium could not assimilate ammonia in the medium that contained sterilized lagoon-extract. The ability of ammonia assimilation might be influenced by some nitrogen compounds, carbon compounds, or other trace elements and by a combination of these compounds in the medium. Hu et al. [6] reported that regulation of the pathway of ammonia assimilation in *Bacillus subtilis* was different from that in enteric bacteria and that the pool size of the intracellular glutamine derived from the assimilated ammonia also differed. Therefore, phylogenetic differentiation of the isolates may influence the amount of ammonia assimilation in the medium.

We had isolated and identified high ammonia-assimilating microorganisms from the waste treatment systems [15, 16]. The diversity and distribution of microbial species of ammonia-assimilating microorganisms was remarkably changed as process proceeded [17]. The isolated species of ammonia-assimilating microorganisms might vary as their isolation source was changed. Some isolates show a high ammonia-assimilating ability in the mixed culture containing PVA-immobilized cells; this ability was similar to that observed in their pure cultures. Although the rates of ammonia consumption in the mixed culture were lower than those in the pure culture of free cells [15, 16], ammonia consumption of the isolates L2015, C10, C24, and II14 was not markedly lowered in the mixed culture containing PVA-immobilized cells in this study. In the mixed culture containing PVA-immobilized cells, they showed the rate of ammonia consumption at the range of 30 to 60% of the value, which they showed in a pure culture with free cells. These results suggested that the isolates could grow dominantly without competition with the other microorganisms in the mixed culture by being immobilized with PVA. Moreover, the results (Figs. 1, 2) indicated that ammonia-assimilating isolates in the immobilized cells could assimilate ammonia even in the presence of the complex microbial community of the lagoon. Thus, these immobilized cells may be one of the candidates that perform the ammonia removal from wastewater in field usage.

The observed surface area of the immobilized cells (Fig. 3b) suggested that the immobilized ammonia-assimilating isolates grew dominantly, while actively assimilating ammonia in absence of competition from other microorganisms. Cell immobilization by nondegradable substances is disadvantageous due to the retention of nitrogen compounds in the compost. However, this conventional technique may contribute to ammonia removal by heterotrophs in livestock manure treatment systems. Although PVA by itself was well-known to be nontoxic to microorganisms [7], some of the immobilized isolates that showed low ammonia-assimilating abilities might be influenced to a greater extent by low concentrations of oxygen and nutrients than by the condition of free cells.

Birrer et al. [1] reported that changes in the ratios of carbon and nitrogen sources affect the production of

PGA by 20 g/l in *B. licheniformis*. Carbon to nitrogen ratio in the medium is known to affect microbial ammonia assimilation and microbial degradation of organic nitrogen [20]. The optimum ratios of high ammonia-assimilating microorganisms in the medium should be clarified in future studies.

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