

**Analysis and application of microbial consortia
involved in ammonification and nitrification
for organic hydroponics**

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ABBREVIATIONS

ABBREVIATIONS

<i>amoA</i>	Ammonium monooxygenase α -subunit
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
bp	Base pair (s)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ENMC	Enriched nitrifying microbial consortia
LB medium	Luria-Bertani medium
NH_4^+	Ammonium ions
NO_2^-	Nitrite ions
NO_3^-	Nitrate ions
NOB	Nitrite-oxidizing bacteria
<i>nxrA</i>	Nitrite oxidoreductase α -subunit
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Real-Time PCR

INTRODUCTION

Nitrogen cycle is one of the most important material cycles in terrestrial ecosystems. Nitrification is a part of nitrogen cycle which generated nitrate as final product (5). In soil, nitrogen exists in various forms, ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-). However, the preference form of nitrogen for plant is nitrate.

In soil culture, organic nitrogen fertilizers are added directly during crop cultivation. The organic nitrogen fertilizers added to soil are degraded via ammonification by the microbial community and subsequent nitrification by nitrifying bacteria, i.e., ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and nitrite-oxidizing bacteria (NOB) (13, 27, 33, 66). These microbial activity plays important roles in the nitrogen cycle (38, 50).

The nitrifying bacteria are key microorganisms to generate nitrate for crop cultivation (5, 15). The studies on the nitrifying microbial consortia and its interaction with the environment are necessary for improvement of nitrification process. However, culture of nitrifying bacteria is difficult because of their sensitivity to many external factors such as pH and temperature change as well as to various organic and inorganic compounds that act as nutrient and energy sources (16, 38, 46, 47, 50). Moreover, the complexity of soil makes it difficult to study nitrifying microbial consortia in environment. Therefore, an efficient method is required to analyze these nitrifying microorganisms.

One of the possible methods to analyze nitrifying microbial consortia is to reconstruct the consortia in water system such as hydroponic system which has low complexity compared to soil. However, the conventional hydroponic system lack of controlling method of microorganisms. The introduction of organic compounds cause uncontrollable growth of microorganisms.

On the other hand, the controlling method of microorganisms in hydroponic system was established using soil as nitrifying microorganism sources. Nitrifying microorganisms in soil were enriched by controlling amounts of organic nitrogen fertilizers under aerobic condition. Organic nitrogen compounds were gradually degraded to nitrate by the enriched nitrifying microbial consortia. Using the culture solution

INTRODUCTION

containing the enriched nitrifying microorganisms enable organic fertilizers to be directly added to the solution during crop cultivation (60).

The organic hydroponic system includes the following two processes. The first is an enrichment process of nitrifying microorganisms. The second process is crop cultivation which maintaining the enriched nitrifying microbial consortia by addition of organic fertilizer.

For successful organic hydroponics, it is necessary to efficiently produce nitrate from organic fertilizer. Therefore, the analysis of the enriched microbial consortia involved in the ammonification and nitrification is required to develop efficient tools for organic hydroponics.

In this study, Firstly, the author investigated the composition transition of nitrifying microorganisms in relation to the nitrogen-ions transitions. Secondly, the author constructed a microbial co-culture to perform ammonification and nitrification. Thirdly, the author isolated the nitrite-oxidizing bacteria existing with commensal denitrifying bacteria from enriched nitrifying microbial consortia. Fourthly, the author assessed the biological safety of the enriched nitrifying microbial consortia.

CHAPTER I

Analysis of microbial community and nitrogen transition with enriched nitrifying soil microbes for organic hydroponics

Hydroponics has been widely used for vegetable cultivation (3, 70). Its advantage includes the easy control of nutrients in the hydroponic solution (12, 22). The application of hydroponics also helps prevent soil-borne diseases, pests, and weeds, which leads to reduced use of herbicides (10, 11, 18, 22, 70). However, in conventional hydroponics, organic fertilizers have phytotoxic effects that lead to poor plant growth (30, 37, 60) and are difficult to use because of the lack of a microbial community that degrades and mineralizes organic nitrogen compounds into nitrate ions (NO_3^-).

In nature, organic nitrogen fertilizers are added directly during crop cultivation. The organic nitrogen fertilizers added to soil are degraded via ammonification by the microbial community and subsequent nitrification by nitrifying bacteria, i.e., ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and nitrite-oxidizing bacteria (NOB) (13, 27, 33, 66). This microbial activity plays important roles in the nitrogen cycle (38, 50).

Culture of nitrifying bacteria is difficult because of their sensitivity to many external factors such as pH and temperature change as well as to various organic and inorganic compounds that act as nutrient and energy sources (16, 38, 46, 47, 50). Therefore, intensive studies on the nitrifying microbial community and its interaction with the environment are necessary for establishing efficient methods to control nitrification processes. However, as the complexity of soil makes it difficult to study nitrifying microbial communities properly, a simple sustainable model system is required to enable us to investigate these nitrifying microbial communities.

Recently, a novel method for organic nitrogen compounds-mineralization in a hydroponic solution using enriched soil microorganisms with high nitrifying activity was established (60). Soil microorganisms were enriched by controlling amounts of organic nitrogen fertilizers under aerobic condition. Using the culture solution containing the enriched nitrifying microorganisms enable organic fertilizers to be directly added to the solution during crop cultivation (36, 60). This organic hydroponic system includes the

following two processes. The first is a microbial enrichment process that gradually degrades organic nitrogen fertilizers to NO_3^- . The second process is crop cultivation using organic fertilizer while maintaining the enriched nitrifying microbial community. In this study, the author investigated the transition of a nitrifying microbial community in relation to the transition of inorganic nitrogen compounds in the first process of the organic hydroponic system, a microbial enrichment process. These results revealing representative microorganisms acting in each ammonification and nitrification stages will be valuable for development of artificial simple microbial consortia for organic hydroponics consisted of identified heterotrophs and autotrophic nitrifying bacteria.

MATERIALS AND METHODS

Evaluation of soils as sources of microorganisms for nitrification in organic hydroponics

Various soils (approximately 50 types) such as paddy soil collected from a demonstration paddy field at Kyoto University, commercial "Nae-ichiban" nursery soil (Sumirin-Nousankougyo, Co., Ltd, Kaifu, Aichi, Japan), Sanyo-bark compost (Sanyo Chip Kogyo, Yamaguchi, Japan) as well as manures (cow, poultry), and fermented leaves, etc. were screened for representative source of microorganisms. Microorganisms were extracted from each soil type (5 g/L) after it was added to paper bag filters and immersed in 800 mL of distilled water for 24 h (soil soak solution). After 24 h, the paper bags were removed from the soil soak solution, and 0.5 g/L of a fish-based soluble fertilizer (total N 7.3%, total C 21.6%, and C/N ratio: 2.9:1) (Cooperatives Yaizu Fisheries Processing Center, Shizuoka, Japan) was added every day to the solution for 5 days. Concentrations of ammonium ions (NH_4^+), nitrite ions (NO_2^-), and nitrate ions (NO_3^-) were monitored using Ammonium-Test (Merck, Frankfurt, Germany), Nitrite-Test (Merck, Frankfurt, Germany), and Nitrate-Test (Merck, Frankfurt, Germany) kits, respectively.

Analysis of microbial community during nitrification process

Microorganism source and culture condition

Sanyo-bark compost, prepared from the bark of the tree, was used as the representative soil microorganism source. The culture medium contained 0.5 g/L of polypeptone NF (Nippon Seiyaku, Tokyo, Japan) and 1 g/L of CaCO₃ in the soil soak solution extracted from the bark compost (5 g/L). The experiments were conducted in test tubes, each containing 15 mL of the medium at 28°C with shaking (300 rpm) under aerobic conditions. The microorganisms were collected from the 5 test tubes (total 75 mL), and the transition of nitrogen ions was monitored until nitrification was complete.

DNA extraction

Cultivation was performed in 10 test tubes, and microorganisms from 5 test tubes that showed the standard nitrogen ions transition were collected by centrifugation at 8,000 rpm for 10 min. The collected microorganisms were used for polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. Total genomic DNA extraction from the collected microorganisms was performed using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan).

PCR amplifications and DGGE analysis

PCR was performed using a T-3000 Thermo cycler (Biometra, Göttingen, Germany). Primer pairs used to analyze communities of prokaryotes (44), particularly AOB (54) and NOB (69), and eukaryotes (44) are shown in Table 1-1. GC-clamped primers were used for PCR-DGGE analysis, and no GC-clamped primers were used for the identification of microbes.

PCR amplification of prokaryotes (the V6-V8 region of prokaryotic 16S rRNA gene) was performed with primers 984F GC and 1378R (Table 1-1). PCR amplification was performed in a reaction mixture (50 µL) containing 1 µL of genomic DNA, 1 µL of KOD FX Neo (Toyobo, Osaka, Japan), 25 µL of 2×PCR buffer, 2 mM of each dNTP, and 0.25 µM of primers. The PCR program was 50 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 1 min.

PCR amplifications for AOB using *amoA* gene primer (amoA1F GC and amoA2IR) target to ammonia monooxygenase of AOB, NOB using *nxrA* gene primer (F2842 F1 nxrA GC and F2843 R2 nxrA) target to nitrite oxidoreductase catalytic subunit of NOB, and eukaryotes using 18S rRNA gene primer (NS1 and GCFung) were performed in a reaction mixture (50 μ L) containing 1 μ g of genomic DNA, 1 μ L of Ex taq HS (Takara-Bio, Shiga, Japan), 25 μ L 2 $\times$ PCR buffer, 2 mM of each dNTP, and 0.25 μ M of primers. The PCR program was performed under the following conditions: AOB: 50 cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 1 min; NOB: 50 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; and eukaryotes: 50 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 1 min.

The PCR products were purified using the High Pure PCR Clean Up Micro Kit (Roche Diagnostics, Switzerland). The purified PCR products (approximately 200 ng) were analyzed by DGGE using the DCode System (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions. For analysis of the prokaryotes, AOB and NOB, 6% of polyacrylamide gels with denaturing solution gradients of 50%-70% (100% denaturing solution contained 40% formamide and 7.0 M urea) were prepared. Electrophoresis was performed with a constant voltage of 50 V at 58°C for 18 h. For analysis of eukaryotes, 7% of polyacrylamide gels with denaturing solution gradients of 20%-45% (100% denaturing solution contained 40% formamide and 7.0 M urea) were prepared. Electrophoresis was performed with a constant voltage of 50 V at 60°C for 20 h. DGGE gels were stained with Ez Stain Silver (ATTO, Tokyo, Japan) and dried using the Gel-Dry Kit (TEFCO, Japan). Markers for DGGE analyses of prokaryote and eukaryote groups were DGGE Marker III and DGGE Marker IV (Nippon Gene, Toyama, Japan), respectively.

Identification of species corresponding to DGGE band

The band was cut with a disposable scalpel (FETHER SAFETY ROZOR, Japan) from DGGE dry gel. In total, 100 μ L of TE buffer was added to the gel and crushed with sterile bio-smasher II (Sarstedt K.K., Japan). A total of 1 μ L of the solution was used as the PCR template. PCR amplification was performed by EX taq HS (Takara-Bio, Shiga, Japan) with the following primers: prokaryotes: 984F and 1378R, AOB: amoA1F and

amoA2IR, NOB: F1370 F1 nxrA and F2843 R2 nxrA; eukaryotes: NS1 and Fung). PCR products were analyzed using the CEQ 2000XL Genetic Analysis System (Beckman Coulter Inc., Fullerton, California). The sequences similarity (%) were analyzed based on BLAST database.

Table 1-1 Primers for PCR-DGGE analysis and sequence analysis

Target	Primer	Nucleotide sequence	T _m (°C)
16S rRNA gene of prokaryote	984F	5'-AACGCGAAGAACCTTAC-3'	55
	984F GC	5'-GCCCCGGGCGCGCCCCGGGCGGGGCGG GGGCACGGGGGAACGCGAAGAACCTTAC-3'	
	1378R	5'-TTCTACTGGTGGT-3'	
Ammonia monooxygenase of AOB	<i>amoA1F</i>	5'-GGGGTTTCTACTGGTGGT-3'	52
	<i>amoA1F</i>	5'-CGCCCCGCCGCGCCCCGCGCCCGTCCCCGCCG	
	GC	CCCCCGCCCGGGGTTTCTACTGGTGGT-3'	
	<i>amoA2IR</i>	5'-CCCCTCIGIAAAGCCTTCTTC-3'	
Nitrite oxidoreductase catalytic subunit of NOB	F1370 F1	5'-CAGACCGACGTGTGCGAAAG-3'	55
	nxrA		
	F2842 F1	5'-CGCCCCGCCGCGCGCGGGCGGGGCGGGG	
	nxrA GC	GCACGGGGGGCAGACCGACGTGTGCGAAAG-3'	
	F2843 R2	5'-TCCACAAGGAACGGAAGGTC-3'	
18S rRNA gene of eukaryote	NS1	5'-GTAGTCATATGCTTGTCTC-3'	50
	GCFung	5'-CGCCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCATTCCCGTTACCCGTTG-3'	
	Fung	5'-CATTCCCCGTTACCCGTTG-3'	

RESULTS

Evaluation of soils as sources of microorganisms for nitrification in organic hydroponics

In this study, various types of soils were evaluated as sources of microorganisms for nitrification in organic hydroponics. It was found that almost all soils were useful as microorganism sources, showing the similar result on nitrification activity in terms of ammonification and nitrification rates and the amount of NO_3^- accumulation. Fig. 1-1 A shows a representative result using "Nae-ichiban" nursery soil as microorganism source. Nitrification completed within 38 days with undetectable amounts of NO_2^- and accumulations of 250 ppm NO_3^- .

The nitrification process performed by microorganisms derived from the Sanyo-bark compost (Fig. 1-1 B) finished about 10 days faster than that performed by microorganisms derived from "Nae-ichiban" nursery soil (a representative result from other soils) (Fig. 1-1 A). Thus, Sanyo-bark compost was selected as the representative microorganism source for further experiments.

During the nitrification process, a wide range of pH change (4.5-9.0) was observed (Fig. 1-1 A and B). In the initial stage of nitrification, NH_4^+ were generated through organic nitrogen compounds degradation. Then, NO_2^- were generated by the oxidation of NH_4^+ , and NO_2^- were further oxidized into NO_3^- . The nitrification process finished when the concentration of NO_2^- fell below the detection limit and the concentration of NO_3^- became constant.

The addition of CaCO_3 (1 g/L) to the organic hydroponic solution resulted in further activation of the nitrification process by maintaining the pH between 6.5 and 8.5 (Fig. 1-1C).

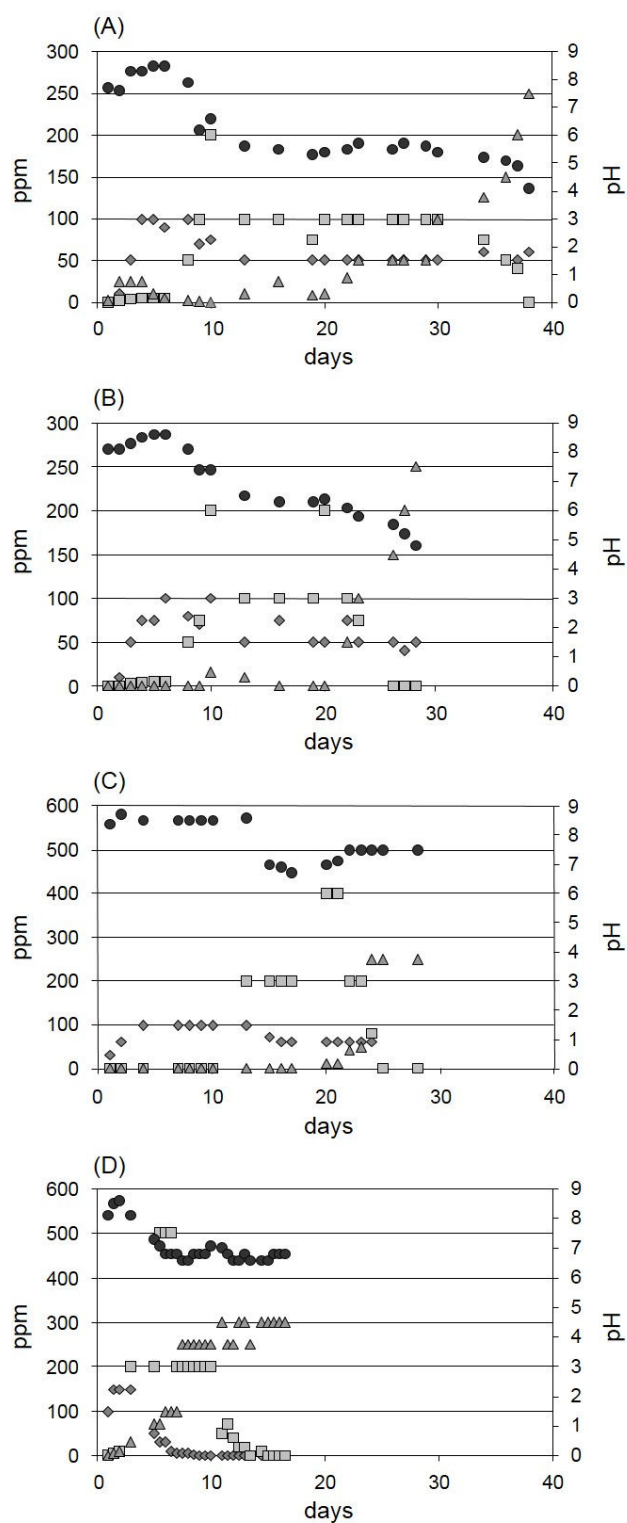


Fig. 1-1 Transition of inorganic nitrogen ions and pH by selected representative microbial sources for the nitrification process "Nae-ichiban" nursery soil (A), Sanyo-bark compost (B), Sanyo-bark compost with calcium carbonate (C), and enriched microbial community with calcium carbonate (D).

$\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^- ; $\bullet$, pH.

Repeated nitrification by enriched nitrifying microorganisms

After the nitrification was finished, the enriched nitrifying microorganisms derived from the Sanyo-bark compost were collected by centrifugation at 8,000 rpm for 10 min and were reused for repeating the nitrification process. Repeated nitrification (about 40 times) revealed a similar transition of inorganic nitrogen ions (i.e. NH_4^+ to NO_2^- and then to NO_3^-), and nitrification finished faster as it was repeated (Fig. 1-1 D).

These results showed that the enriched microbial community enhanced and stabilized nitrification activity and was useful as a seed culture for further nitrification.

Analysis of microbial community during the nitrification process

The transition of microorganisms during nitrification in the course of the enrichment from the bark soil was investigated. To obtain normalized results, experiments were performed using 10 test tubes, and microorganisms from 5 test tubes showing standard nitrogen ion transition were collected. The genome of collected microorganisms were extracted and analyzed by PCR-DGGE. The transition of inorganic nitrogen ions and pH were measured.

As shown in Fig. 1-2, NH_4^+ were detected at day 2 of the nitrification process, with a constant concentration at 80 ppm. NO_2^- were detected at day 12 of the nitrification process, and the NO_3^- concentration increased up to 160 ppm at day 16. The level of NO_2^- became less than the detection limit at day 20 of the nitrification process. The conversion of NO_2^- to NO_3^- was observed during days 16 to 19 of the nitrification process. The level of NO_2^- started to decrease when NO_3^- were detected. The result was reproducible when using the enriched microbes.

The V6-V8 region of prokaryotic 16S rRNA gene was analyzed by PCR-DGGE (Fig. 1-3 B). The variety of microorganisms derived from the soil and soil soak solution showed high similarity. The profile of the observed band was changed during nitrification process and could be divided into three different parts. The first stage of the DGGE profile was NH_4^+ generation. The second stage was NO_2^- generation. The last stage was NO_3^- accumulation. It was observed that the variety of microorganisms was dramatically decrease when NO_2^- was detected (Fig. 1-3 A and B) and the variety was increased again after NO_2^- disappeared (Fig. 1-3 A). The bands were cut off from the gel and used as PCR

templates for amplification of a 470-bp fragment of prokaryotic 16S rRNA gene and the sequences of the amplified products were analyzed. Bands A1-A11 were close to *Bacillus cereus* (Fig. 1-3 B, A1-A2), *Bacillus* sp. (Fig. 1-3 B, A3-A4), *Lysobacter* sp. (Fig. 1-3 B, A5), *Pseudomonas* sp. (Fig. 1-3 B, A6), *P. montelii* (Fig. 1-3 B, A7), *Pseudomonas* sp. (Fig. 1-3 B, A8, A10), *P. putida* (Fig. 1-3 B, A9), and *Acinetobacter* sp. (Fig. 1-3 B, A11). All sequences showed above 98% similarity.

The genera *Bacillus*, *Lysobacter* and *Pseudomonas* were observed throughout the NH_4^+ generation stage. They once decreased in the beginning of NO_2^- accumulation, but increased again with decrease of NO_2^- . On the other hand, the genus *Acinetobacter* sp. (Fig. 1-3 B, A11) was found only in middle NH_4^+ generation stage and disappeared when NO_2^- started to accumulate (Fig. 1-3 A). This strain could be a nitrification supporting strain in that specific stage of NH_4^+ generation. These results also suggested that the common soil bacteria such as *Bacillus* and *Pseudomonas* were the major bacteria acting throughout the nitrification process.

DGGE based on *amoA* gene (ammonia oxygenase) of AOB was performed during the nitrification process (Fig. 1-3 C). The profile of the observed band showed difference of the band in NH_4^+ generation stage and NO_2^- accumulation stage (Fig. 1-3 A and C). The DGGE band profile indicated no significant change of the variety of microorganisms during NH_4^+ generation stage until NO_2^- generation (Fig. 1-3 A and C). The bands were cut off from the gel and used as PCR templates for amplification of a 491-bp fragment of the *amoA* gene in AOB and the sequences of the amplified products were analyzed. The analyzed bands (B1-B6) were close to *Nitrosomonas nitrosa* (Fig. 1-3 C, B1, B3-5), uncultured AOB (Fig. 1-3 C, B2), and uncultured *Nitrosomonas* sp. (Fig. 1-3 C, B6). All sequences showed above 98% similarity.

The results suggested that NH_4^+ oxidation was mainly performed by the genus *Nitrosomonas*. *Nitrosomonas nitrosa* (Fig. 1-3 C, B1) and uncultured AOB (Fig. 1-3 C, B2) found during NO_2^- accumulation stage were purposed as NO_2^- tolerant strain and supported NH_4^+ oxidation process after NO_2^- accumulation.

NOB was analyzed by DGGE using *nxrA* gene (nitrite oxidoreductase) through the nitrification process (Fig. 1-3 D). The profile of the observed band showed difference of the band in NH_4^+ generation stage and NO_2^- accumulation stage (Fig. 1-3 A and D).

The bands were cut off from the gel and used as PCR templates for amplification of a 322-bp fragment of the *nxrA* gene in NOB and the sequences of the amplified products were analyzed. The analyzed bands (C1-C7) were close to *Nitrobacter vulgaris* (Fig. 1-3 D, C1), *Nitrobacter winogradskyi* (Fig. 1-3 D, C2-C6), and uncultured *Nitrobacter* sp. (Fig. 1-3 D, C7). All sequences showed above 98% similarity.

The result showed that NO_2^- oxidation was mainly performed by *N. winogradskyi*. The genus *Nitrobacter* became dominant in NO_2^- accumulation stage showed the probability for NO_2^- tolerance and could be useful for supporting nitrification process in a high NO_2^- concentration condition.

DGGE analysis of the eukaryote community during the nitrification process was performed using the 18S rRNA gene (Fig. 1-3 E). The bands were cut off from the gel and used as PCR templates for amplification of a 390-bp fragment of the eukaryotic 18S rRNA gene and the sequences of the amplified products were analyzed. The analyzed bands (D1-D6) were close to *Cercomonas* sp. (Fig. 1-3 E, D1), *Mortierella alpina* (Fig. 1-3 E, D2-D3), *Leuconeuropa pulcherrima* (Fig. 1-3 E, D4), *Penicillium oxalicum* (Fig. 1-3 E, D5), and *Hartmannella vermiformis* (Fig. 1-3 E, D6). All sequences showed above 98% similarity.

The result showed that *L. pulcherrima* (Fig. 1-3 E, D4) and *H. vermiformis* (Fig. 1-3 E, D6) were observed in NH_4^+ generation stage and NO_2^- generation stage. On the other hand, *Cercomonas* sp. (Fig. 1-3 E, D1), and *M. alpina* (Fig. 1-3 E, D2-D3) were detected only when NO_2^- accumulated (Fig. 1-3 A). Most of eukaryote disappeared with increase of NO_3^- .

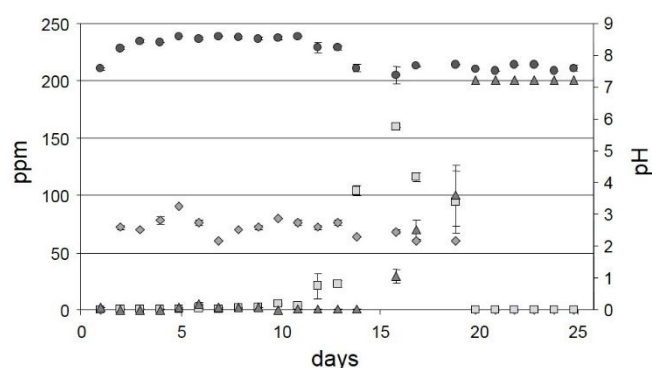


Fig. 1-2 Monitoring of inorganic nitrogen and pH transition during the nitrification process. The nitrification process was performed in test tubes with polypeptone NF and soil soak solution as a microbial source at 28°C, 300 rpm. ♦, NH_4^+ ; ■, NO_2^- ; ▲, NO_3^- ; ●, pH.

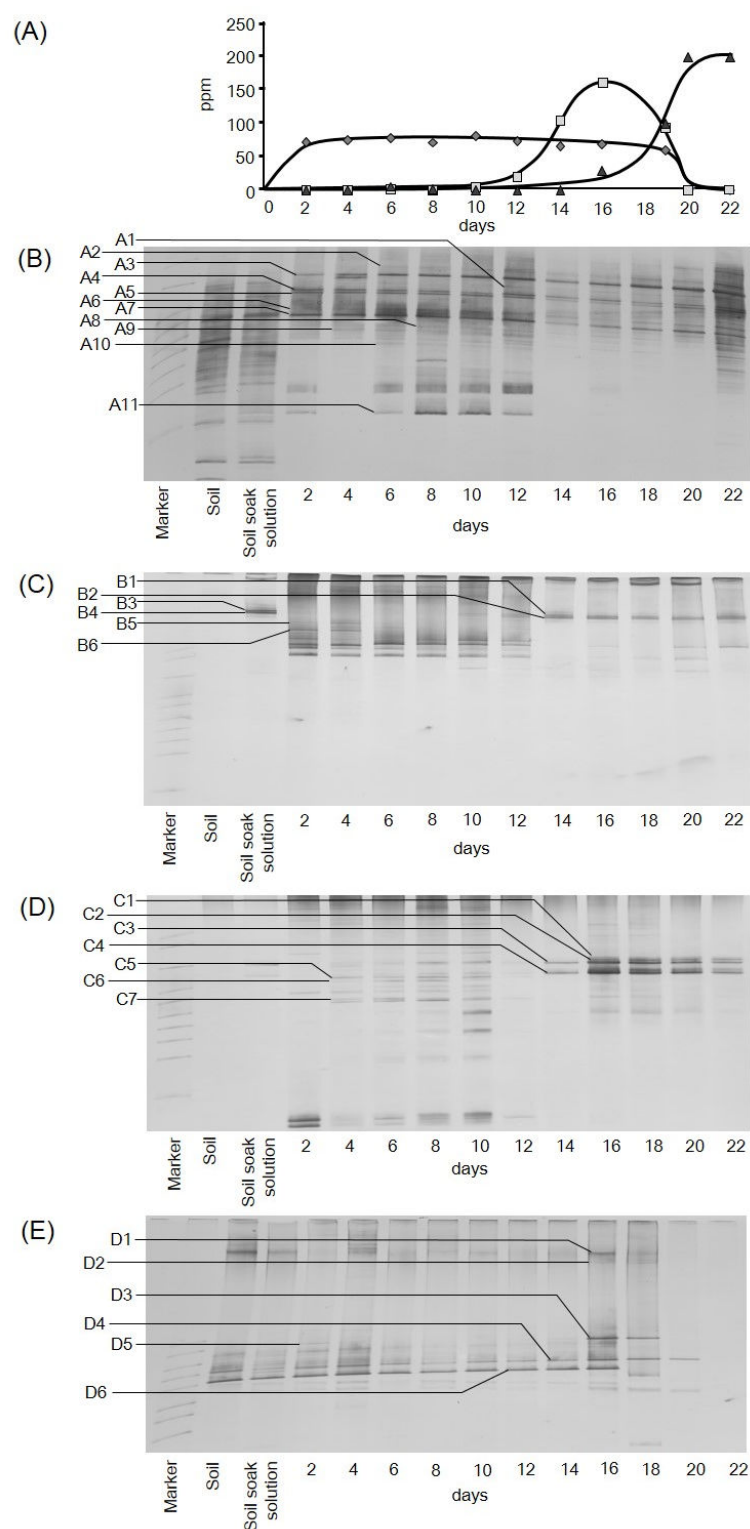


Fig. 1-3 PCR-DGGE analysis of microorganisms and nitrogen transition during the nitrification process. $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^- (A), DGGE analysis of prokaryote community transition (B), ammonia-oxidizing bacterial community transition (C), nitrite-oxidizing bacterial community transition (D), eukaryote community transition (E).

DISCUSSION

Recently, the demand for organic hydroponic-grown crops has rapidly increased because of concerns regarding the security and safety of foods as well as environmental conservation (24). The organic hydroponic method is an environmentally friendly technique and is useful as a sustainable production system in future because it decreases environmental stresses and enables efficient crop and vegetable cultivation. For successful organic hydroponics, it is necessary to produce NO_3^- from organic fertilizer using microorganisms efficiently.

Enriched nitrifying microorganisms from Sanyo-bark compost allowed a stable and accelerated organic nitrogen transformation to NO_3^- and provided an example of environmental information for controlling natural nitrifying microorganisms. The nitrification activity performed by microorganisms derived from Sanyo-bark compost was further enhanced by the addition of CaCO_3 which maintained the pH between 6.5 and 8.5 (Fig. 1C). The pH value is one of the most significant factors affecting the nitrification rate (29, 58). This findings correlated with those of Strauss *et al.* (2002) (64), who reported that pH had a strong effect on the nitrification process. Nitrification rates were the highest at pH 7.5 and decreased with declining and increasing pH (64). Nitrification performed by an enriched culture of nitrifiers obtained from wastewater was inhibited at pH values below 5.8 (48). The C/N ratio of the organic fertilizer is another factor affecting the nitrification process. The fish-based soluble fertilizer using in this study had C/N ratio of 2.9. An organic fertilizer with a high C/N ratio (more than 11) may lead to inadequate nitrogen supply to microorganisms (60, 64).

The present analysis of a microbial community demonstrated that the microbial community during the nitrification process in a water system was simpler than soil microbes and consisted of a few microorganisms of a certain types (Fig. 3A and B). This finding suggested that the simple enriched microbial community can be used in the ammonification and nitrification for organic hydroponics. PCR-DGGE analysis showed that genera *Nitrosomonas* and *Nitrobacter* were mainly detected during NH_4^+ and NO_2^- oxidation stage, respectively (Fig. 3A, C, and D). It has been proposed that the free ammonia concentration of approximately 4 ppm or above led to inhibit nitrite-oxidizing activity of NOB (2, 31). On the other hand, the culture solution in this study contained

higher concentrations of ammonium ion (approximately 100 ppm), indicating that nitrifying bacteria was enriched and performed nitrification even under such conditions.

The populations of heterotrophic and autotrophic nitrifying bacteria in organic soils has been studied and revealed that the populations of heterotrophic nitrifiers was approximately 100 times higher than that of autotrophic nitrifying bacteria. In contrast, the nitrification activity in soil was mainly performed by autotrophic nitrifying bacteria (65). In this studies, the microbial consortia derived from soil without enrichment performed nitrification within 1 month or more (Fig. 1A-C). However, the enriched microbial consortia performed nitrification within 2 weeks (Fig. 1D), showing the possibility that autotrophic nitrifying microorganisms were enriched. This is under investigation by metagenomic analysis.

In addition, the genera *Nitrosomonas* and *Nitrobacter* are known as the most abundant nitrifiers responsible for nitrification in the wastewater treatment system (23, 32, 46, 47, 68). However, *Nitrosomonas* and *Nitrobacter* were difficult to isolate and cultivate (16, 38, 46, 47, 50), thus making it difficult to artificially construct model systems consisting of culturable strains. On the other hand, this results implied an interaction between heterotrophic bacteria and these unculturable strains. For example, *Bacillus*, *Lysobacter*, *Pseudomonas*, and *Leuconeurospora*, which are observed throughout the nitrification process, are assumed to support ammonification and nitrification activity of autotrophic nitrifying bacteria. The strains found only in a specific stage, e.g., *Acinetobacter* sp. in middle NH_4^+ generation (Fig. 3A and B) and *Cercomonas* and *Mortierella* in NO_2^- accumulation (Fig. 3A and E), are also useful for controlling the specific stages.

In terms of pathogenicity, crops cultivated in organic hydroponics could suppress root-borne diseases. Previous studies revealed that the organic hydroponic system can potentially control *Fusarium* and bacterial wilt disease (11, 18, 19).

These finding provided fundamental information to construct simple microbial consortia for ammonification and nitrification useful for organic hydroponics supported by interaction between heterotrophs and autotrophic nitrifying bacteria.

SUMMARY

Nitrifying microbial consortia were enriched from bark compost in a water system by regulating the amounts of organic nitrogen compounds and by controlling the aeration conditions with addition of CaCO_3 for maintaining suitable pH. Repeated enrichment showed reproducible mineralization of organic nitrogen via the conversion of ammonium ions (NH_4^+) and nitrite ions (NO_2^-) into nitrate ions (NO_3^-).

The change in microbial composition during the enrichment was investigated by PCR-DGGE analysis with a focus on prokaryote, ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and eukaryote cell types. The microbial transition had a simple profile and showed clear relation to nitrogen ions transition. *Nitrosomonas* and *Nitrobacter* were mainly detected during NH_4^+ and NO_2^- oxidation, respectively.

These results revealing representative microorganisms acting in each ammonification and nitrification stages will be valuable for development of artificial simple microbial consortia for organic hydroponics consisted of identified heterotrophs and autotrophic nitrifying bacteria.

CHAPTER II

Construction of a microbial co-culture useful for ammonification and nitrification

In natural environment, almost all organic compounds are degraded by heterotrophic microorganisms via ammonification and the resultant ammonium ion is subsequently transformed to nitrate via two-step nitrification reaction by chemoautotrophic nitrifying microorganisms (25, 58, 67), ammonia-oxidizing bacteria or archaea (1, 27, 33, 66), and nitrite-oxidizing bacteria (9, 67). These microorganisms are known to play important roles in nitrogen cycle as well as for plant growth.

Until now, hydroponic culture system have been developed and widely used for crop cultivation (70). However, organic fertilizers cannot be directly applied to hydroponic system. Introduction of organic compounds causes uncontrollable growth of microorganisms because lack of controlling method of microorganisms in hydroponic culture solution (30, 37, 60).

Recently, a novel method for organic hydroponics have been developed. The method includes introduction of controlled amounts of organic fertilizer and soil as a microbial source under aerobic condition into hydroponic solution. Using the culture solution containing the enriched nitrifying microorganisms enable organic fertilizers to be directly added to the culture solution during hydroponic cultivation (60).

Most of autotrophic nitrifying bacteria were unable to utilize organic nitrogen compounds (38, 50). Therefore, it is important to focus the interaction between heterotrophs and these autotrophs for controlling the mineralization of organic nitrogen compounds. However, the complexity of soil makes it difficult to study nitrifying microbial communities properly. Therefore, a simple sustainable model system is required to enable us to investigate these nitrifying microbial communities. Moreover, these nitrifying bacteria are hard to cultivate because of the sensitivity to many factors such as organic compounds as well as pH and temperature (38, 46, 47, 50).

Therefore, the purpose of this research is to construct a simple co-culture only with three strains, a heterotroph, an ammonia-oxidizing bacterium, and a nitrite-oxidizing bacterium, as a microbial interaction model for ammonification and nitrification. *Nitrosomonas* and *Nitrobacter* are well known as the most abundant autotrophic nitrifying

bacteria responsible for nitrification particularly in a waste water treatment system. Moreover, in previous study, the author also revealed that *Nitrosomonas* and *Nitrobacter* were mainly detected during NH_4^+ and NO_2^- oxidation in the enrichment of nitrifying microbial consortia for organic hydroponics (55). Thus, in this study, *N. europaea* and *N. winogradskyi* were used as representative nitrifying bacteria for co-culture evaluation. On the other hand, some heterotrophic bacteria were reported to have denitrification activity and a NO_2^- oxidation even under aerobic condition (6, 35, 40, 52, 53). Therefore, the author considered that it was necessary to evaluate NO_2^- conversion by heterotrophic bacteria for construction of co-culture. Heterotrophs were screened according to following steps. First, heterotrophic bacteria were selected based on denitrification and nitrite oxidation activity in nutrient medium with high concentration of NO_2^- . Subsequently, co-cultivation with a selected heterotroph and nitrifying bacteria, *Nitrosomonas europaea* NBRC 14298 and *Nitrobacter winogradskyi* NBRC 14297 was performed in nutrient medium. Third, the ability of the co-culture was investigated under various cultivation conditions. Furthermore, the transition of nitrifying bacteria in the co-culture was analyzed during ammonification and nitrification of organic nitrogen compounds.

MATERIALS AND METHODS

Bacteria strain

Fifty-four heterotrophic bacteria (Table 2-1), *Nitrosomonas europaea* NBRC 14298 (ATCC 19718) and *Nitrobacter winogradskyi* NBRC 14297 (ATCC 14123) were purchased from type culture collections (NBRC, NITE Biological Resource Center, Chiba, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan; and ATCC, American Type Culture Collection, Rockville, Md, USA).

Table 2-1 Nitrite conversion by heterotrophic bacteria

Group	Species	Strain	NH ₄ ⁺ (ppm)	NO ₂ ⁻ (ppm)	NO ₃ ⁻ (ppm)
A	<i>Arthrobacter globiformis</i>	NBRC 12137	80	-200	300
	<i>Arthrobacter oxydans</i>	NBRC 12138	80	-230	400
	<i>Arthrobacter oxydans</i>	NBRC 108611	5	-130	200
	<i>Arthrobacter sulfureus</i>	NBRC 12678	70	-230	400
	<i>Arthrobacter ureafaciens</i>	NBRC 12140	60	-230	250
	<i>Bacillus badius</i>	NBRC 15713	80	-50	40
	<i>Citricoccus</i> sp.	NBRC 3763	60	-230	300
	<i>Kocuria rhizophila</i>	NBRC 12780	80	-220	300
	<i>Micrococcus luteus</i>	NBRC 3066	70	-230	300
	<i>Micrococcus luteus</i>	NBRC 3067	80	-130	200
	<i>Micrococcus luteus</i>	NBRC 3333	60	-230	300
	<i>Pimelobacter simplex</i>	NBRC 12679	5	-150	150
B	<i>Bacillus amyloliquefaciens</i>	NBRC 3007	30	-80	40
	<i>Bacillus amyloliquefaciens</i>	NBRC 3022	30	-80	50
	<i>Bacillus amyloliquefaciens</i>	NBRC 3037	30	-130	70
	<i>Bacillus firmus</i>	NBRC 3330	10	-80	50
	<i>Bacillus licheniformis</i>	JCM 20329	30	-130	50
	<i>Bacillus megaterium</i>	NBRC 12108	30	-130	80
	<i>Bacillus pumilus</i>	NBRC 12092	30	-130	25
	<i>Bacillus subtilis</i>	NBRC 3009	30	-80	50
	<i>Brevibacterium aurantiacum</i>	NBRC 12171	70	-130	70
	<i>Brevibacterium iodinum</i>	NBRC 3558	80	-80	30
	<i>Brevibacterium linens</i>	NBRC 12141	10	-130	40
	<i>Brevibacterium linens</i>	NBRC 12170	70	-130	80
	<i>Brevundimonas diminuta</i>	NBRC 3140	30	-80	40
	<i>Curtobacterium pusillum</i>	NBRC 12674	5	-80	25
	<i>Desemzia incerta</i>	JCM 1969	60	-80	50
	<i>Flavobacterium lutescens</i>	NBRC 3084	30	-130	70
	<i>Lysinibacillus fusiformis</i>	NBRC 15717	60	-150	100
	<i>Lysinibacillus sphaericus</i>	NBRC 3525	80	-150	100
	<i>Lysinibacillus sphaericus</i>	NBRC 3526	60	-130	80
	<i>Lysinibacillus sphaericus</i>	NBRC 3341	80	-150	100
	<i>Microbacterium esteraromaticum</i>	JCM 9172	5	-80	25
	<i>Micrococcus luteus</i>	ATCC 10240	60	-130	40
	<i>Pseudomonas fluorescens</i>	NBRC 3925	70	-230	100
	<i>Pseudomonas fragi</i>	NBRC 3458	60	-220	20
	<i>Pseudomonas pavonaceae</i>	NBRC 12052	60	-130	10
	<i>Pseudomonas putida</i>	NBRC 3738	70	-130	10
	<i>Pseudomonas putida</i>	NBRC 12653	70	-130	20
	<i>Pseudomonas putida</i>	NBRC 12996	70	-180	20
	<i>Pseudomonas</i> sp.	NBRC 12691	30	-150	40
	<i>Pseudomonas taetrolens</i>	NBRC 3460	80	-150	25
C	<i>Alcaligenes faecalis</i>	NBRC 13111	80	0	0
	<i>Arthrobacter protophormiae</i>	NBRC 12128	30	0	0
	<i>Brevibacillus parabrevis</i>	NBRC 3331	60	0	0
	<i>Cellulomonas biazotea</i>	NBRC 12680	30	0	0
	<i>Corynebacterium paurometabolum</i>	NBRC 16120	5	0	0
	<i>Hydrogenophilus thermoluteolus</i>	NBRC 14593	30	0	0
	<i>Kocuria</i> sp.	NBRC 3064	30	0	0
	<i>Methylobacterium extorquens</i>	NBRC 3708	30	0	0
	<i>Planomicroacterium keanokoites</i>	NBRC 12536	60	0	0
	<i>Pseudomonas chlororaphis</i>	NBRC 3904	60	0	0
	<i>Rhodobacter sphaeroides</i>	NBRC 12203	30	0	0
	<i>Sinomonas atrocyanea</i>	NBRC 12956	30	0	0

Evaluation of denitrification activity and NO_2^- conversion by heterotrophic bacteria

The evaluation of denitrification activity and NO_2^- conversion by heterotrophic bacteria was conducted in according to the method reported by Sakai et al, 1996 (56). Heterotrophic bacteria were cultivated in the medium [(w/v) 0.03% NaNO_2 , 1% glucose, 1% peptone, 0.1% yeast extract, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5] at 28°C. Inorganic nitrogen ions transition was monitored for 7 days by using Ammonium-Test, Nitrite-Test, and Nitrate-Test (Merck, Frankfurt, Germany).

Co-cultivation of heterotrophs and nitrifying bacteria

Co-cultivations were performed under the following conditions. The selected heterotrophic bacteria were pre-cultivated by shaking (120 rpm) in the NBRC 802 medium [(w/v) 1% polypepton, 0.2% yeast extract, and 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0] at 28°C for one day.

N. europaea NBRC 14298 was prepared in the modified HPEPS medium [(w/v) 0.125% $(\text{NH}_4)_2\text{SO}_4$, 0.05% KH_2PO_4 , 1.192% HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid) (Dojindo molecular technologies, Inc., Kumamoto, Japan), 0.05% NaHCO_3 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.0075% Fe-EDTA, pH 7.8-8.0] at 28°C for 5 days.

N. winogradskyi NBRC 14297 was prepared according to Boon and Laudelout's medium (4) [(w/v) 0.238% NaNO_2 , 0.32% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.0272% KH_2PO_4 , 0.0001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.6] at 28°C for 10 days.

Subculture grown of *N. europaea* and *N. winogradskyi* were used for inoculation into the culture solution. The volume of the inoculum for sub-culturing was 20% of medium.

Co-cultivation inoculated with 80 μL (1% v/v, approximately 10^6 cell/mL of nitrifying bacteria) of each pre-cultured medium was carried out by shaking at 28°C, 120 rpm in 20 ml Erlenmeyer flask containing 8 ml following medium (Table 2-2); Medium type 1 [(w/v) 0.078% $(\text{NH}_4)_2\text{SO}_4$, 0.1% glucose, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$], Medium type 2 [(w/v) 1% peptone, 0.1% yeast extract, 1% glucose, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$], and Medium type 3 [(w/v) 0.1% peptone, 0.01% yeast extract, 0.1% glucose, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$]. The amount of the nitrogen

compositions of medium type 2 was as same as that of NO_2^- conversion evaluation. The C/N ratio of these medium was adjusted at 2.4. Total N concentration of medium type 2 was 10 times higher than the others. The amounts of NO_2^- and NO_3^- from pre-cultured medium carried over to fresh medium was low (NO_2^- , 10 ppm, NO_3^- , 25 ppm). Inorganic nitrogen ions transition was monitored for 15 days.

Table 2-2 Cultivation medium for co-culture

Composition (g/L)	Medium type 1	Medium type 2	Medium type 3
$(\text{NH}_4)_2\text{SO}_4$	0.78	-	-
Peptone	-	10.0	1.0
Yeast extract	-	1.0	0.1
Glucose	1.0	10.0	1.0
K_2HPO_4	2.0	2.0	2.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1	0.1	0.1
Total nitrogen	0.165	1.65	0.165
C/N ratio	2.4	2.4	2.4

Effect of various conditions on ammonification and nitrification by the co-culture

The co-culture, *B. badius* NBRC 15713 and nitrifying bacteria, was carried out in the co-cultivation medium under various following conditions at 28°C by shaking (120 rpm). Nitrogen ions transition was monitored by using Ammonium-Test, Nitrite-Test, and Nitrate-Test (Merck, Frankfurt, Germany) in all experiments.

The initial pH value was adjusted to 5-10 by 1N HCl or NaOH solution. The culture medium containing [(w/v) 0.1% Polypepton NF (Nihon, Pharmaceutical co., Ltd., Tokyo, Japan), 0.3% glucose, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (C/N ratio 10)].

The media composed of various organic nitrogen sources, Hipolypepton N, Hipolypepton S (Nihon, Pharmaceutical co., Ltd., Tokyo, Japan), Bacto peptone, Bacto tryptone, Bacto yeast extract (Becton, Dickinson and company, MD, USA), and Casamino Acids “DAIGO” (Nihon, Pharmaceutical co., Ltd., Tokyo, Japan) were prepared at the same concentration (0.1%, w/v). The culture medium containing [(w/v) 0.1% organic nitrogen source, 0.3% glucose, 0.2% K_2HPO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$].

The media with different C/N ratio were prepared with two organic nitrogen sources, Polypepton NF (Nihon, Pharmaceutical co., Ltd., Tokyo, Japan) and bonito extract (Cooperatives Yaizu Fisheries Processing Center, Shizuoka, Japan) at 120 mg-total N and adjusted to C/N ratio 10 and 50 by addition of glucose. The culture medium containing [(w/v) 120 mg-total N of organic nitrogen source, 0.3% and 1.5% glucose, 0.2% K₂HPO₄ and 0.01% MgSO₄•7H₂O].

The media with the different amount of nitrogen concentration was prepared with Polypepton NF (0.1-10 g/L) and was adjusted to C/N ratio 10 by addition of glucose. The culture medium containing [(w/v) 0.1-10% Polypepton NF, 0.3-3% glucose, 0.2% K₂HPO₄ and 0.01% MgSO₄•7H₂O].

The population transitions in the co-culture during ammonification and nitrification

Co-cultivation condition

The co-culture, *B. badius* NBRC 15713 and nitrifying bacteria, was cultivated in a 2 L Erlenmeyer flask containing 500 ml of the co-cultivation medium [(w/v) 0.1% Polypepton NF, 0.3% glucose, 0.2% K₂HPO₄ and 0.01% MgSO₄ • 7H₂O (C/N ratio 10), pH 7.5] at 28°C by shaking (120 rpm). The sampling culture solution was monitored the transition of inorganic nitrogen ions, OD, and pH for 14 days. Genomic DNA was extracted from culture solution (1 ml) using NucleoSpin® Microbial DNA kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) for subsequent RT-PCR. DNA concentration was measured spectrophotometrically using NanoDrop 2000 spectrophotometer (Thermo Scientific, Kanagawa, Japan).

Real-time PCR assay

Real-time PCR quantification was carried out using LightCycler II instrument (Roche Diagnostics GmbH, Germany). Amplification reaction was performed with the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan). Primer sets used for real-time PCR assay are shown in Table 2-3.

Quantification of the 16S rRNA gene of total bacteria was performed using the primers 1055f (17) and 1392r (21) (Table 2-3). Quantification of the 16S rRNA gene of

N. europaea NBRC 14298 was performed using the two forward primers CTO198f A/B and CTO189f C, one reverse primer CTO654r (34). Quantification of the 16S rRNA gene *N. winogradskyi* NBRC 14297 was performed using the primers FGPS872 and FGPS1269' (14). The PCR mixture contained 2 µL of DNA, 10 µL of KOD SYBR qPCR Mix, and 4 pmol of primers. The real-time PCR thermocycling steps for all primer sets were 95°C for 2 min, and 50 cycles at 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min.

As a standard, tenfold serial dilution of the plasmids carrying the standard sequence, ranging from 10¹-10⁹ serial dilution were used. Plasmid DNA pUC118-Hinc II/BAP vector (Mighty Cloning Reagent Set, Blunt End; Takara Bio Inc. Shiga, Japan) carrying a 16S rRNA gene was extracted with a QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) and the plasmid concentration (nanograms per microliter) was measured spectrophotometrically using NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA).

All experiments were carried out in triplicate, and the averages of three separate experiments are presented in the figures.

Table 2-3 Primer set for real-time PCR analysis

Target gene	Primer	Nucleotide sequence	Reference
16S rRNA ammonia-oxidizing bacteria	CTO189f	GGAGRAAAGCAGGGGATCG	Kowalchuk et al. (1997)
	A/B*		
	CTO189f C*	GGAGGAAAGTAGGGGATCG	Kowalchuk et al. (1997)
	CTO654r	CTAGCYTTGTAGTTTCAAACGC	Kowalchuk et al. (1997)
16S rRNA <i>Nitrobacter</i> sp.	FGPS872	TTTTTTGAGATTGCTAG	Degrange and Bardin (1995)
	FGPS1269'	CTAAAACTCAAAGGAATTGA	Degrange and Bardin (1995)
Bacterial 16S rRNA	1055f	ATGGCTGTCGTCAGCT	Ferris et al. (1996)
	1392r	ACGGGCGGTGTGTAC	Ferris et al. (1996)

* A mixture of CTO 189f A/B and CTO 189f C at the ratio of 2:1 were used as the forward primers

RESULTS

Evaluation of denitrification activity and NO_2^- conversion by heterotrophic bacteria

Among 54 bacteria strains cultured in the nutrient medium containing nitrite (0.03% NaNO_2 , w/v), 42 strains showed NO_2^- oxidation ability to the NO_3^- (Table 2-1).

They could be divided into three types, the group A (12 strains) with NO_2^- oxidation to NO_3^- almost stoichiometrically, the group B (30 strains) with NO_2^- conversion without NO_3^- accumulation, and the group C (12 strains) with neither NO_2^- conversion nor NO_3^- accumulation.

The nitrogen loss during NO_2^- oxidizing to NO_3^- were observed among the group B, indicating that NO_2^- was reduced to gaseous compounds via denitrification, or converted via assimilative nitrite reduction to ammonium ion. Group C strains were considered to have no NO_2^- oxidation activity. Therefore, group A strains which showed no denitrification activity and high nitrite oxidizing activity were considered to support nitrification process and selected as candidates for co-culture.

Evaluation of co-culture with heterotrophs and nitrifying bacteria

First, each heterotrophic bacterium of group A was cultivated in three types medium composed of inorganic nitrogen (type 1), high concentration of organic nitrogen (type 2), and low concentration of organic nitrogen (type 3) to investigate ammonification and nitrification. As a result, neither nitrite nor nitrate ions accumulation were observed in these media by each strain (data not shown). The co-cultivation only with *Nitrosomonas europaea* NBRC 14298 and *Nitrobacter winogradskyi* NBRC 14297 as a control showed nitrification activity only in type 1 medium contained ammonium sulfate (Table 2-4).

Co-cultivations in medium type 1

At day 3, co-cultures of respective heterotrophic bacteria *Arthrobacter globiformis* NBRC 12137, *A. oxydans* NBRC 12138, 108611, *Bacillus badius* NBRC 15713, *Citricoccus* sp. NBRC 3763, *Kocuria rhizophila* NBRC 12780, *Micrococcus luteus* NBRC 3066, *M. luteus* NBRC 3333 with nitrifying bacteria showed higher NO_2^-

accumulation than that of control consisted of only nitrifying bacteria in medium type 1 (Table 2-4). The author considered that these heterotrophs supported ammonia oxidation activity.

At day 6, co-cultures of respective heterotrophic bacteria *A. globiformis* NBRC 12137, *A. oxydans* NBRC 12138, 108611, *B. badius* NBRC 15713, *Citricoccus* sp. NBRC 3763, *K. rhizophila* NBRC 12780, *M. luteus* NBRC 3066, 3333 with nitrifying bacteria showed higher NO_3^- accumulation than that of control, indicating that these heterotrophic bacteria enhanced nitrification.

At 15 day, co-cultures of *A. globiformis* NBRC 12137, *A. oxydans* NBRC 108611, *B. badius* NBRC 15713, *Citricoccus* sp. NBRC 3763, *K. rhizophila* NBRC 12780, and *M. luteus* NBRC 3333 performed complete nitrification with approximately 200-400 ppm NO_3^- accumulation. In contrast, control did not complete nitrification (approximately 100 ppm NO_2^- remaining). These results suggested that NO_2^- oxidation activity of these heterotrophic bacteria supported nitrification process (Table 2-4).

Co-cultivations in medium type 2

Medium type 2 was the same organic nitrogen composition of Sakai's medium except for NaNO_2 (56). No effective nitrification activity was observed by any co-cultures (Table 2-4). The author considered that organic nitrogen concentration was too high for autotrophic bacteria resulting in inhibition of nitrifying bacterial growth. Thus, the author prepared medium type 3 composed of one tenth amount of organic nitrogen source compared with type 2.

Co-cultivations in medium type 3

At day 6, co-cultures of *A. oxydans* NBRC12138, *A. sulfureus*, *B. badius* and *M. luteus* NBRC 3067 showed considerable amount of NO_2^- accumulation (Table 2-4). However, at day 15, only the co-culture of *B. badius* NBRC 15713 showed the effective ammonification and nitrification with approximately 400 ppm NO_3^- accumulation without NO_2^- remaining (Table 2-4). As a result, the author focused the co-culture of *B. badius* with nitrifying bacteria.

CHAPTER II

Table 2-4 Inorganic nitrogen ions accumulate in three type medium

day 0		Medium type 1 (ppm)				Medium type 2 (ppm)				Medium type 3 (ppm)			
*Species	Strain	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
		200	10	25	7.5	0	10	25	7.5	0	10	25	7.5
day 3		NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
<i>Arthrobacter globiformis</i>	NBRC 12137	80	40	25	7.5	7.5	7	20	7.5	2.5	3	25	7.5
<i>Arthrobacter oxydans</i>	NBRC 12138	90	30	25	7.5	5	40	0	7.5	7.5	5	12.5	7.5
<i>Arthrobacter oxydans</i>	NBRC 108611	75	30	25	7.5	5	7	17.5	7	0	3	25	7.5
<i>Arthrobacter sulfureus</i>	NBRC 12678	80	3	25	7	25	1	25	8.5	0	10.5	12.5	7.5
<i>Arthrobacter ureafaciens</i>	NBRC 12140	75	2	25	7	15	5	5	6	7.5	2	25	7.5
<i>Bacillus badius</i>	NBRC 15713	70	40	25	7.5	30	10	25	8.5	10	50	25	7.5
<i>Citricoccus sp.</i>	NBRC 3763	80	60	25	7.5	25	0	20	7.5	5	9	25	7.5
<i>Kocuria rhizophila</i>	NBRC 12780	70	30	25	7.5	20	0	25	7	5	4.5	25	7.5
<i>Micrococcus luteus</i>	NBRC 3066	75	60	25	7.5	0	8.5	20	7.5	15	4	25	7.5
<i>Micrococcus luteus</i>	NBRC 3067	90	25	25	7.5	70	3.5	25	9	15	30	25	7.5
<i>Micrococcus luteus</i>	NBRC 3333	100	60	25	7.5	30	0	25	8	0	20	25	7.5
<i>Pimelobacter simplex</i>	NBRC 12679	80	3	25	7	0	2	20	6	10	0	25	7
Control		100	20	25	7.5	0	8	10	7.5	0	2	25	7.5
day 6		NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
<i>Arthrobacter globiformis</i>	NBRC 12137	30	25	225	6	5	7	20	7.5	2.5	1.5	25	7.5
<i>Arthrobacter oxydans</i>	NBRC 12138	65	275	65	7	30	10	15	8	0	155	20	7.5
<i>Arthrobacter oxydans</i>	NBRC 108611	30	225	200	6.5	5	6	20	7.5	0	0	27.5	7.5
<i>Arthrobacter sulfureus</i>	NBRC 12678	65	11	45	7	70	5	35	8	0	140	27.5	7.5
<i>Arthrobacter ureafaciens</i>	NBRC 12140	70	2.5	30	7	20	0	12.5	5.5	2.5	1	27.5	7.5
<i>Bacillus badius</i>	NBRC 15713	45	325	85	6.5	30	17.5	20	8.5	0	325	50	7.2
<i>Citricoccus sp.</i>	NBRC 3763	30	400	75	6.5	45	0	17.5	8	5	17.5	27.5	8
<i>Kocuria rhizophila</i>	NBRC 12780	30	250	60	6.5	30	0	20	8.5	2.5	17.5	25	8
<i>Micrococcus luteus</i>	NBRC 3066	65	300	65	6.5	0	8.5	20	7.5	20	30	27.5	8
<i>Micrococcus luteus</i>	NBRC 3067	65	40	45	7	100	2	20	9	20	135	27.5	8
<i>Micrococcus luteus</i>	NBRC 3333	65	300	175	6.5	45	0	20	7.5	0	4	30	8
<i>Pimelobacter simplex</i>	NBRC 12679	55	2	25	7	0	1	20	5.5	7.5	0	25	7
Control		60	300	40	6.8	0	8	10	7.5	0	0	35	7.5
day 9		NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
<i>Arthrobacter globiformis</i>	NBRC 12137	60	0	400	6	5	7	20	7.5	2.5	1.5	30	7.5
<i>Arthrobacter oxydans</i>	NBRC 12138	80	155	225	6.5	30	45	0	8.5	0	200	100	7.5
<i>Arthrobacter oxydans</i>	NBRC 108611	60	0	400	6	0	8.5	20	7.5	0	0	35	7.5
<i>Arthrobacter sulfureus</i>	NBRC 12678	80	7.5	50	7	65	5	30	7.5	0	200	150	7.5
<i>Arthrobacter ureafaciens</i>	NBRC 12140	70	4	30	7	15	0	20	6	2.5	1	27.5	7.5
<i>Bacillus badius</i>	NBRC 15713	65	25	400	6.5	30	20	22.5	8.5	0	300	225	7.2
<i>Citricoccus sp.</i>	NBRC 3763	85	300	225	6.5	65	0	20	8.5	5	17.5	30	8
<i>Kocuria rhizophila</i>	NBRC 12780	65	200	200	6.5	65	0	20	8.5	2.5	22.5	30	8
<i>Micrococcus luteus</i>	NBRC 3066	100	400	175	6.5	65	7	20	7.5	20	40	40	8
<i>Micrococcus luteus</i>	NBRC 3067	100	20	150	7	100	2	20	9	20	160	40	8
<i>Micrococcus luteus</i>	NBRC 3333	100	25	400	6.5	0	0	20	7.5	0	12.5	40	8
<i>Pimelobacter simplex</i>	NBRC 12679	100	2	25	7	0	1	20	6	5	0	25	7
Control		100	350	50	6.5	0	8	10	7.5	0	0	35	7.5
day 12		NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
<i>Arthrobacter globiformis</i>	NBRC 12137	60	0	400	6	5	5	22.5	7.8	2.5	1	40	8
<i>Arthrobacter oxydans</i>	NBRC 12138	75	125	225	6.5	25	50	0	9	0	165	175	7.5
<i>Arthrobacter oxydans</i>	NBRC 108611	60	0	400	6	2.5	4.5	20	7.8	0	0	55	8
<i>Arthrobacter sulfureus</i>	NBRC 12678	80	10	50	7	30	0	20	7	0	145	175	7.5
<i>Arthrobacter ureafaciens</i>	NBRC 12140	75	3.5	30	7	20	0	20	6	2.5	1	40	7.8
<i>Bacillus badius</i>	NBRC 15713	70	0	400	6.5	20	40	22.5	8.5	0	175	275	7
<i>Citricoccus sp.</i>	NBRC 3763	75	100	350	6.5	60	0	20	8.5	5	18.5	32.5	8
<i>Kocuria rhizophila</i>	NBRC 12780	65	150	225	6.5	60	0	20	8.5	2.5	20	30	8
<i>Micrococcus luteus</i>	NBRC 3066	75	375	200	6.5	0	6	20	7.5	0	45	40	8
<i>Micrococcus luteus</i>	NBRC 3067	85	60	150	7	90	2	20	8.5	20	160	45	8
<i>Micrococcus luteus</i>	NBRC 3333	75	0	400	6.5	75	0	20	8	20	18.5	40	8
<i>Pimelobacter simplex</i>	NBRC 12679	100	1.5	27.5	7	0	0	20	6	5	0	25	7.5
Control		150	300	100	6.5	0	7	15	7.5	0	0	40	7.5
day 15		NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH	NH ₄ ⁺	NO ₂ ⁻	NO ₃ ⁻	pH
<i>Arthrobacter globiformis</i>	NBRC 12137	60	0	400	6	5	5	22.5	7.5	2.5	1	40	8
<i>Arthrobacter oxydans</i>	NBRC 12138	75	125	225	6.5	20	45	0	9	0	145	175	7.5
<i>Arthrobacter oxydans</i>	NBRC 108611	60	0	400	6	2.5	3.5	22.5	7.8	0	0	55	8
<i>Arthrobacter sulfureus</i>	NBRC 12678	75	10.5	65	7	20	0	20	7	0	100	225	7.5
<i>Arthrobacter ureafaciens</i>	NBRC 12140	70	1	30	7	20	0	20	6	2.5	1	40	7.8
<i>Bacillus badius</i>	NBRC 15713	65	0	400	6.5	20	40	22.5	8.5	0	0	400	7
<i>Citricoccus sp.</i>	NBRC 3763	75	0	400	6.5	60	0	20	8.5	2.5	50	35	8
<i>Kocuria rhizophila</i>	NBRC 12780	60	0	300	6.5	60	0	20	8.5	2.5	22.5	30	8
<i>Micrococcus luteus</i>	NBRC 3066	75	325	200	6.5	0	6	20	7.5	20	35	40	8
<i>Micrococcus luteus</i>	NBRC 3067	75	35	200	7	70	2	20	8.5	2.5	135	65	8
<i>Micrococcus luteus</i>	NBRC 3333	70	0	400	6.5	60	0	20	8	0	25	40	8
<i>Pimelobacter simplex</i>	NBRC 12679	100	0	27.5	7	0	0	20	6	5	3.5	25	7.5
Control		100	100	250	6.5	0	5	15	7.5	0	0	40	7.5

*Each co-culture was performed with listed heterotrophs, *Nitrosomonas europaea* NBRC 14298, and *Nitrobacter winogradskyi* NBRC 14297.

Control means co-culture only with *N. europaea* and *N. winogradskyi*.

Effect of various conditions on ammonification and nitrification with the co-culture

Effect of initial pH of nutrient medium

Ammonification and nitrification with the co-culture was investigated in nutrient medium adjusted pH value from 5 to 10. The effective nitrification was observed at pH 8-9 and completed within 10 days (Fig. 2-1). On the other hand, no nitrification was observed in the medium pH below 6. In the medium pH 10, nitrite oxidizing activity was affected and nitrification was completed within 13 days. As the pH value of unadjusted co-cultivation medium was 7.5, the author did not adjust pH in subsequent research.

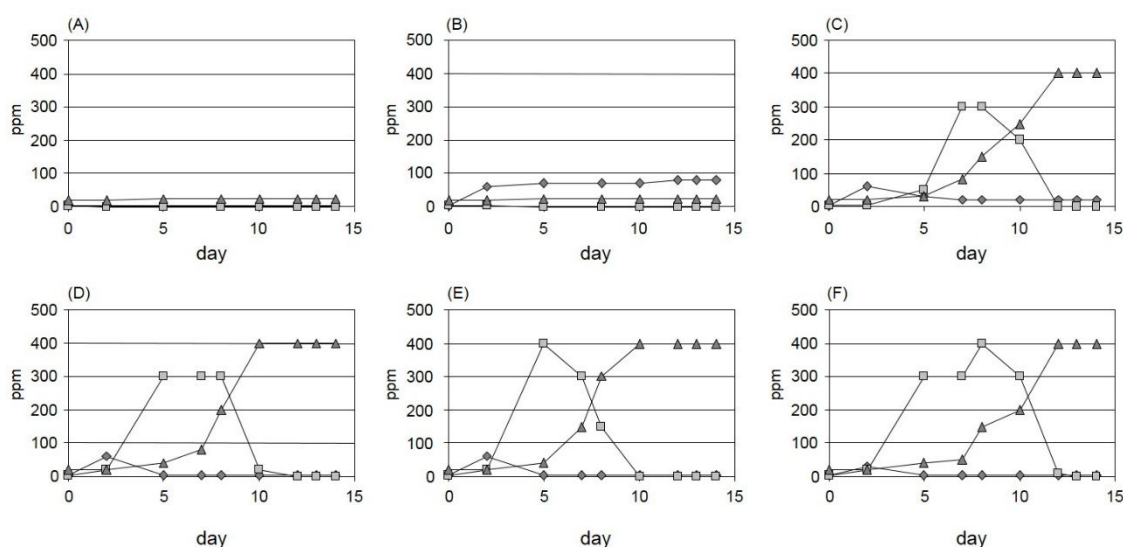


Fig. 2-1 The nitrogen ion transition effected by the initial pH values. A, pH 5; B, pH 6; C, pH 7; D, pH 8; E, pH 9; F, pH 10, $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^-

Effects of various organic nitrogen compounds

Various organic nitrogen compounds prepared from fish, soybean, milk, and yeast extract were used as organic nitrogen sources. As a result, in all conditions except for yeast extract, ammonification and nitrification was performed effectively and completed within 8 days (Fig. 2-2).

CHAPTER II

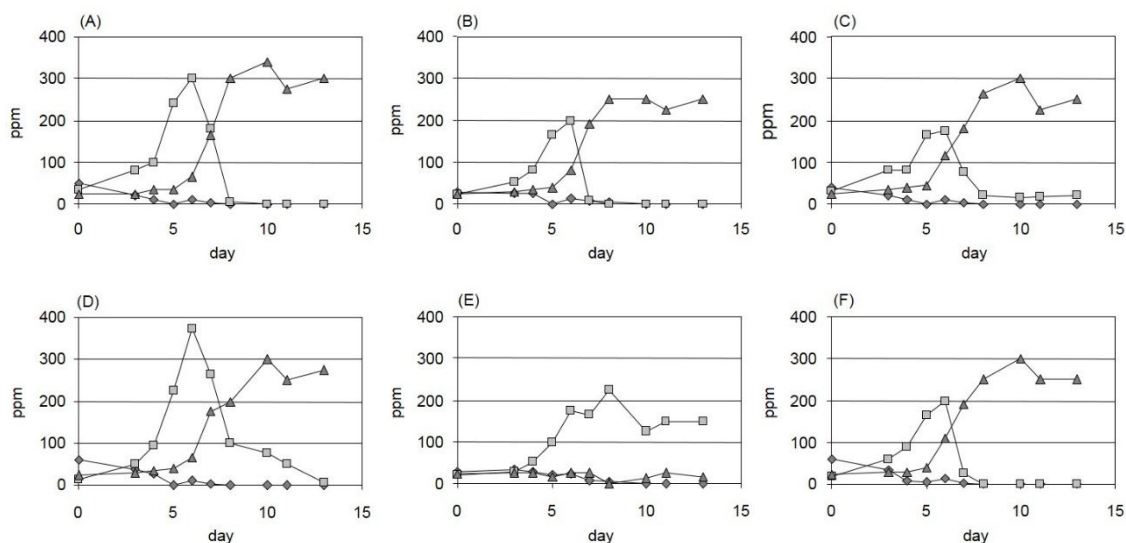


Fig. 2-2 The nitrogen ion transition effected by various organic nitrogen compounds, A, Hipolypepton N; B, Hipolypepton S; C, Bacto peptone ; D, Bacto tryptone; E, Bacto yeast extract; F, Casamino Acids, $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^-

Effect of C/N ratio

Nutrient medium were composed of bonito extract and Polypepton NF as organic nitrogen source and adjusted C/N ratio at 10 and 50 by addition of glucose. As a result, the co-culture completed nitrification in the medium containing Polypepton NF and bonito extract within 13-15 days and 17-20 days under both C/N ratio 10 and 50, respectively (Fig. 2-3) with approximately 400 ppm NO_3^- accumulations.

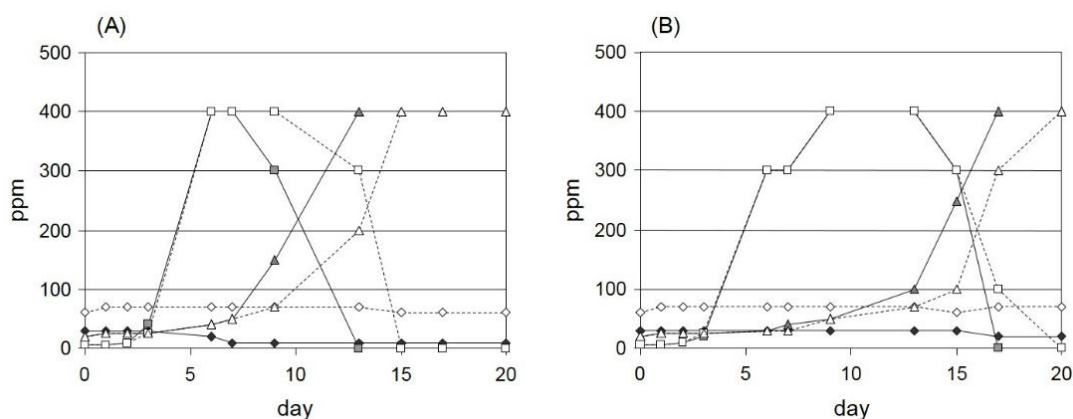


Fig. 2-3 The nitrogen ion transition effected by C/N ratio. A, Polypepton NF; B, Bonito extract, $\blacklozenge$, NH_4^+ C/N 10; $\blacksquare$, NO_2^- C/N 10; $\blacktriangle$, NO_3^- C/N 10; $\diamond$, NH_4^+ C/N 50; $\square$, NO_2^- C/N 50; $\triangle$, NO_3^- C/N 50

Effect of organic nitrogen compound concentrations

The effects of organic compound concentrations (0.1-10 g/L) on ammonification and nitrification by the co-culture were investigated. NO_3^- accumulation was increased gradually with increasing initial concentration of Polypepton NF and was up to 350 ppm in the medium containing 1 g/L Polypepton NF (Fig. 2-4). No nitrite oxidizing activity was observed in the medium containing 2-5 g/L of Polypepton NF although considerable amount of NO_2^- was accumulated. This result suggested that the concentration of organic compounds more than 2 g/L inhibited *N. winogradskyi* activity. Therefore, nitrifying bacteria were inoculated in the media, in which only *B. badius* was cultivated for 2 days. As a result, the nitrification was finished in the media containing 2 g/L (Fig. 2-5).

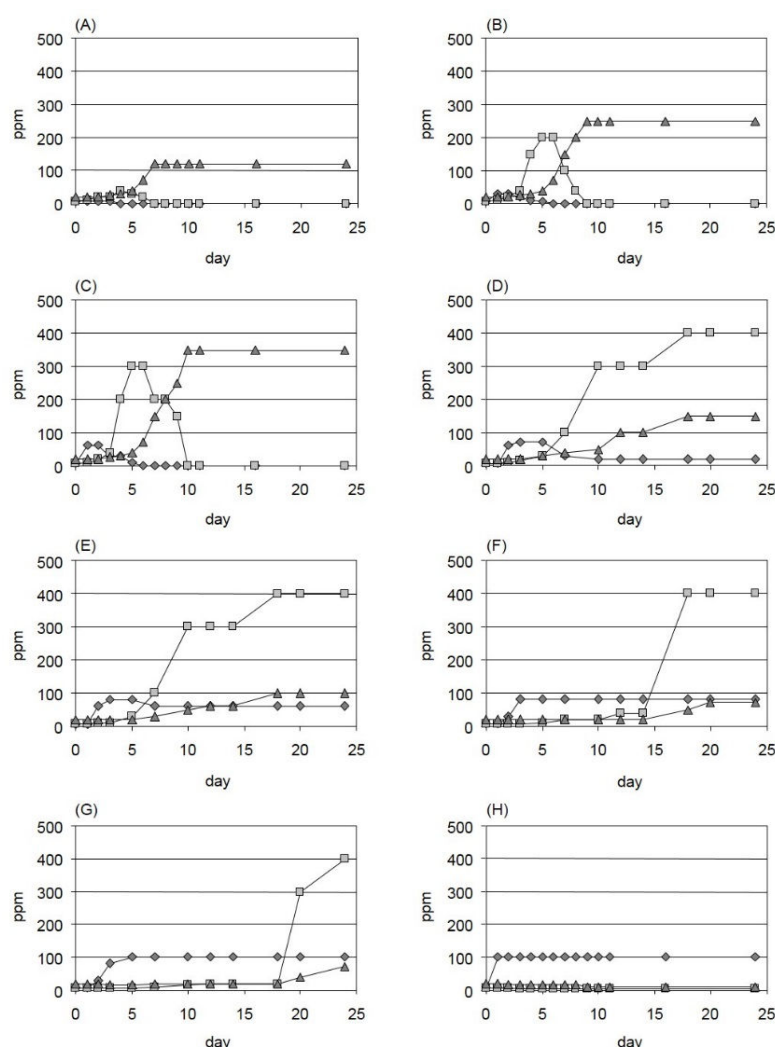


Fig. 2-4 The nitrogen ion transition effected by organic nitrogen compound concentrations. A; 0.1 g/L; B, 0.5 g/L; C, 1 g/L; D, 2 g/L; E, 3 g/L; F, 4 g/L; G, 5 g/L; H, 6 g/L, $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^-

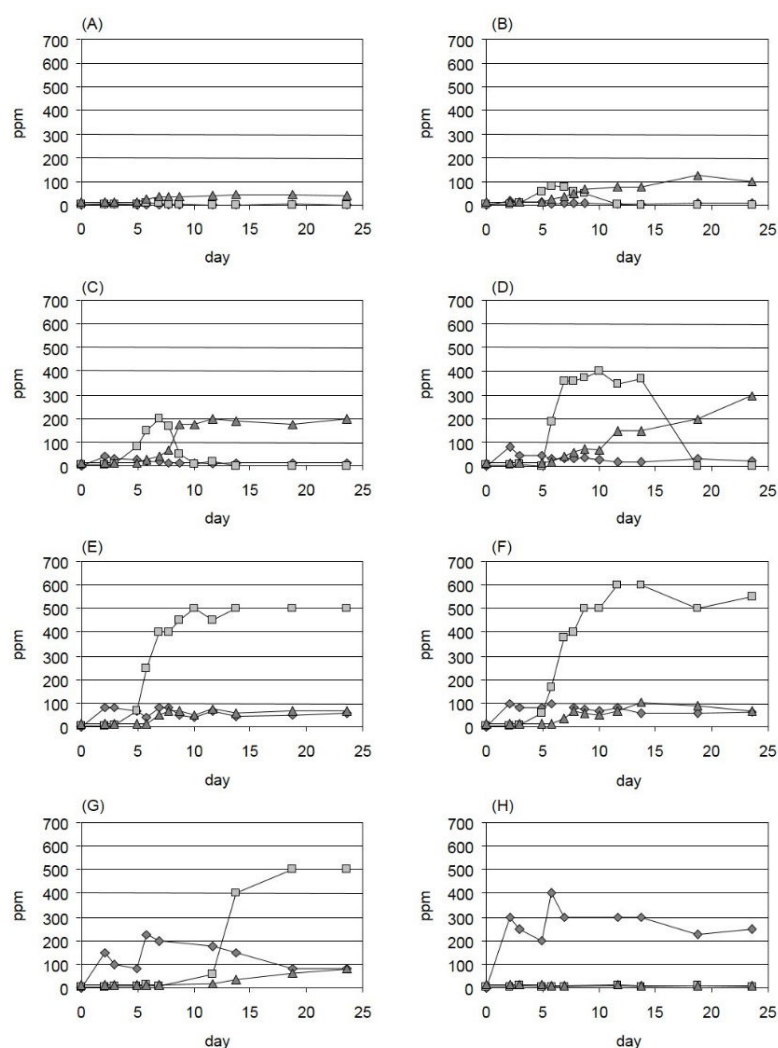


Fig. 2-5 The nitrogen ion transition effected by organic nitrogen compound concentrations with inoculation of nitrifying bacteria after inoculation of *Bacillus badius* for 2 days. A; 0.1 g/L; B, 0.5 g/L; C, 1 g/L; D, 2 g/L; E, 3 g/L; F, 4 g/L; G, 5 g/L; H, 6 g/L; $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^-

Population transition of nitrifying bacteria in the co-culture

For monitoring the population transition of nitrifying bacteria in the co-culture by RT-PCR, the co-cultivation were performed by 2 L Erlenmeyer flask containing 500 ml co-culture medium. The same transition of inorganic nitrogen-ions compared with 8 ml medium volume in 20 ml Erlenmeyer flask was observed. *B. badius* grew vigorously after inoculation for one day and decreased gradually. The pH value of nutrient medium was slightly increased during the ammonification process and gradually decline during nitrification process. The ammonification and nitrification completed for 11 days. The

efficiency of inorganic nitrogen recovery rate was up to 47% (Nitrate-N 56 mg) in the medium containing Polypepton NF (Total-N 120 mg).

Genomic DNA for RT-PCR was extracted from culture solution periodically. RT-PCR was performed with specific primers to nitrifying bacteria (Table 2-3). As a result, RT-PCR revealed that the population transition of nitrifying bacteria showed clear relation to the concentration transition of nitrite and nitrate ions (Fig. 2-6). The specific growth rate of *N. europaea* and *N. winogradskyi* showed 1.53 day^{-1} and 0.65 day^{-1} , respectively.

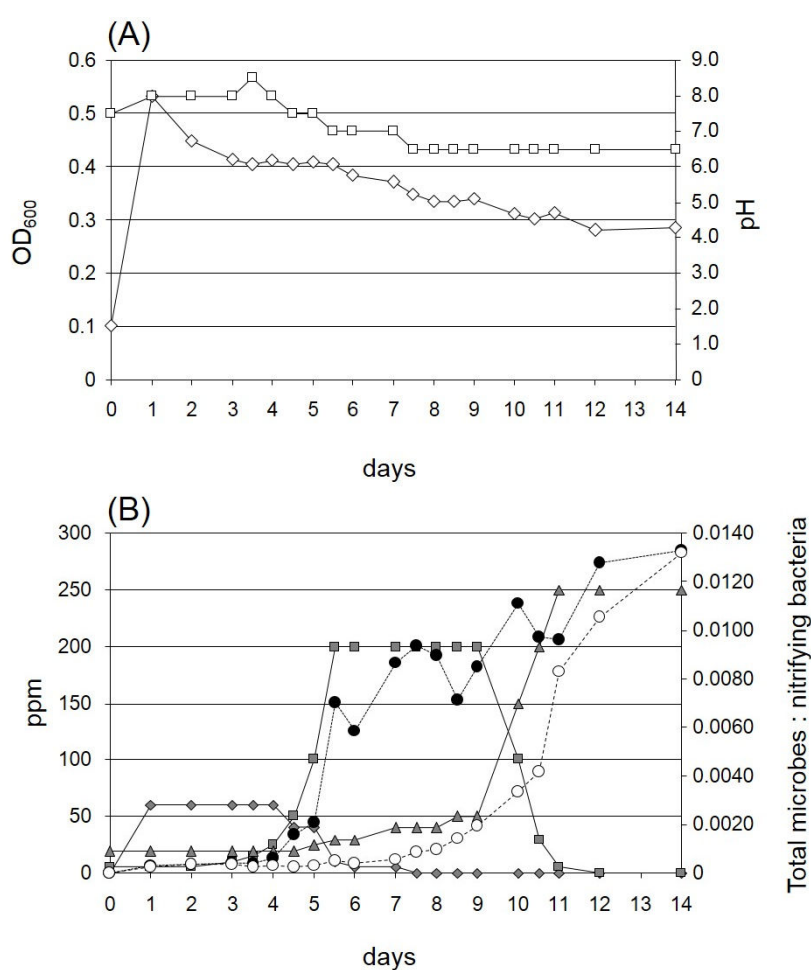


Fig. 2-6 The OD and pH transition (A) and population transition of nitrifying bacteria and nitrogen ions transition (B) during ammonification and nitrification by the co-culture. ◇, OD; □, pH; ◆, NH₄⁺; ■, NO₂⁻; ▲, NO₃⁻; ●, *Nitrosomonas europaea*; ○, *Nitrobacter winogradskyi*

Repeated ammonification and nitrification by the co-culture

After ammonification and nitrification was completed, the co-culture was collected by centrifugation at 8,000 rpm (5,800 g) for 10 min. and reused for mineralization in the fresh medium. Repeated ammonification and nitrification completed in only 4 days and showed inorganic nitrogen-ions transition without NO_2^- accumulation (Fig. 2-7). Furthermore, after this mineralization was completed, the same amount of organic nitrogen compound was added to the culture solution at day 4 and 10, respectively. As a result, a similar transition of inorganic nitrogen ions without NO_2^- accumulation was observed in both adding times of organic nitrogen compound.

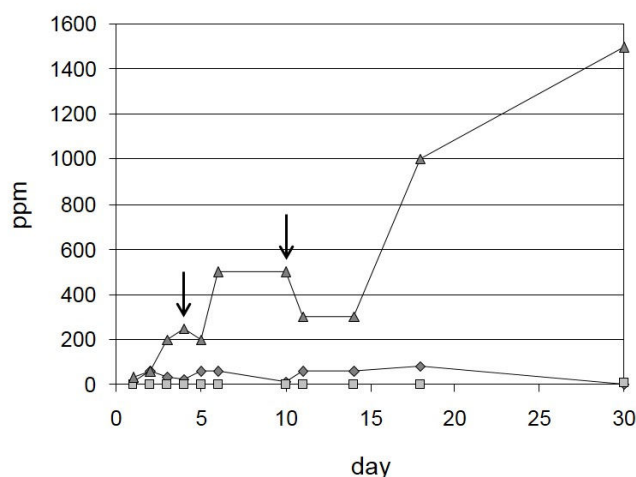


Fig. 2-7 The repeating ammonification and nitrification by the co-culture. $\blacklozenge$, NH_4^+ ; $\blacksquare$, NO_2^- ; $\blacktriangle$, NO_3^- . The arrow is organic nitrogen compounds adding time.

DISCUSSION

In this study, the author addressed to construct artificial ammonification and nitrification model with heterotrophs and nitrifying bacteria under aerobic condition. Until now, the co-culture of *Nitrosomonas* and *Nitrobacter* in the medium containing $(\text{NH}_4)_2\text{SO}_4$ as inorganic nitrogen substrate was studied (55). Thus, the author considered that ammonium ions supplied by heterotroph via degradation of organic nitrogen compounds enable to perform ammonification and nitrification even in a nutrient medium. Moreover, some heterotrophs have ammonia-oxidizing and nitrite-oxidizing activity. These microbes could be a supporting strains not only for ammonification but also

nitrification reaction. On the other hand, denitrification activity was also undesirable feature for ammonification and nitrification of organic nitrogen compounds. Therefore, the author evaluated denitrification activity and nitrite conversion ability of 54 heterotrophs, based on the report by Sakai et al. (56), in a nutrient medium containing high concentration of nitrite. The result of nitrite-oxidizing activity by some heterotrophic bacteria strains (Table 2-1) corresponded with Sakai's (56) results. 12 strains (group A) which showed no denitrification and NO_2^- oxidation to NO_3^- in an equal or more amount were selected as candidates for co-culture. The co-culture of these strains were able to grow in the medium type 1 containing inorganic nitrogen and enhanced nitrification compared with control (Table 2-4). Co-cultures in medium type 2 containing high concentration of organic nitrogen could not perform ammonification and nitrification because organic compounds affected nitrifying bacteria activity (Table 2-4). However, even in medium type 3 containing one tenth concentration of organic nitrogen of medium type 2, only the co-culture of *B. badius* and nitrifying bacteria showed effective ammonification and nitrification. These results indicated that heterotrophic nitrite-oxidizing activity showed no relation with nitrification supporting activity (Table 2-4). Thus, the screening of wide range of heterotrophs will be necessary.

The effect of pH on nitrification has been studied in some conditions such as in aquatic sediments, wastewater system and pure culture. For instance, Struss et al. (2002) (64) revealed that in stream sediments, nitrification rate were highest at pH 7.5 and decreased more gradually with declining pH value than with increasing pH value. Nitrification was inhibited at pH value below 5.8 performed with an enriched culture of nitrifiers obtained from wastewater (48). In this study, the initial pH affected the nitrification performed by the co-culture (Fig. 2-1). Nitrification activity was highest at pH value 8.0-9.0 and gradually decreased with declining pH value and inhibited at pH value below 6.0. These results corresponded with the previous reports (48, 64).

Some studies reported that various kinds of organic fertilizers, corn oil cake, fish flour, canola oil cake, and food waste, were available for ammonification and nitrification by enriched nitrifying microbial consortia (30, 60, 63). The co-culture also showed the ability of ammonification and nitrification of some kinds of organic nitrogen compounds except for yeast extract. Appropriate strains for degradation various kinds of organic

compounds would allow us to design microbial consortia on demand.

Generally, under high environmental C/N, heterotrophic bacteria compete for nitrogen compounds with nitrifying bacteria, resulting in lower nitrification rate (63, 64). It was reported that nitrification activity is depend on NH_4^+ concentration when C/N ratio below 20 and regulated by organic carbon when C/N ratio above 20 in aquatic sediment (64). Shinohara et al. (2011) (60) also suggested that the C/N ratio below 11 should be used for NO_3^- generation by enriched nitrifying microbial consortia. On the other hands, it was considered that the ammonification and nitrification under C/N ratio 50 was due to the slow assimilation of organic nitrogen by *B. badius*

It was reported that the growth of chemolithoautotrophic ammonia-oxidizing bacteria and nitrite-oxidizing bacteria are inhibited by the presence of organic compounds (28, 36, 62). Thus, control of the concentration of organic compound is one of important factors for NO_3^- generation in organic hydroponic solution (60). In this study, the effect of organic compound concentrations experiment also demonstrated that organic compounds (2-10 g/L polypepton NF) led to inhibition of the nitrification, resulting NH_4^+ and NO_2^- accumulation (Fig. 2-4). Moreover, NO_2^- accumulation indicated that *N. winogradskyi* are more sensitive to the organic nitrogen compounds than *N. europaea*. In fact, separated inoculation of nitrifying bacteria achieved ammonification and nitrification even in a nutrient medium containing 2 g/L organic nitrogen (Fig. 2-5). On the other hands, repeated ammonification and nitrification completed in only 4 days without NO_2^- accumulation (Fig. 2-7). This result suggested that *N. europaea* activity is a bottleneck in ammonification and nitrification by the co-culture. This phenomenon was corresponding with previous work (41).

Real-Time PCR assay was widely used and applied for quantification of nitrifying bacteria in wastewater treatment plant (21, 26, 39). However, using of RT-PCR to investigate the change of nitrifying bacterial communities through nitrification process has rarely been reported. The present study revealed the clear relation on the growth of nitrifying bacteria and the concentration transition of nitrite and nitrate ions in a nutrient medium. This result indicated that the nitrification in the co-culture was mainly performed by *Nitrosomonas* and *Nitrobacter* activities.

In the synthetic medium, the specific growth rate of *Nitrosomonas* sp. and *Nitrobacter* sp. was reported approximately 1.24-1.58 day⁻¹ and 0.6-1.4 day⁻¹, respectively (49). The author found that the specific rate of nitrifying bacteria in the co-culture showed same level or above even in a medium containing organic nitrogen. Furthermore, repeated ammonification and nitrification by the co-culture showed possibility to multiply nitrifying bacteria.

In this study, the author succeeded to construct an artificial ammonification and nitrification model. These finding will be a valuable information for development of organic hydroponics as well as for approach to nitrogen cycle in the complex natural environment.

To date single strain and reaction has been studied as a target in applied microbiology. Recently, the analysis of functions based on biological interaction has attracted many attentions. To analyze the complex nature, it would be becoming an important approach to simplify the complexity of nature without loss of the function appeared by biological interaction.

SUMMARY

A microbial co-culture which consisted of identified heterotrophic bacteria and *Nitrosomonas europaea* NBRC 14298 (ATCC 19718) and *Nitrobacter winogradskyi* NBRC 14297 (ATCC 14123) was constructed for ammonification and nitrification of organic nitrogen under aerobic condition. The author evaluated fifty-four heterotrophic bacteria of denitrification activity in a medium contained high nitrite concentration. The strains which showed nitrite-oxidizing activity without denitrification activity were selected as candidates for co-cultivation.

The co-culture of *Bacillus badius* NBRC 15713 with nitrifying bacteria performed ammonification and nitrification effectively under following conditions; pH range 7-10, various kind of organic nitrogen (derived from fish, soybean, and milk), C/N ratios range 10 to 50, and organic nitrogen compound concentration below 0.2%. When the co-culture was performed in the medium containing Polypepton NF (Total-N 120 mg, C/N ratio 10), the efficiency of inorganic nitrogen recovery rate was up to 47% at 28°C

for 11 day. The population transition of nitrifying bacteria by RT-PCR showed clear relation to the concentration transition of nitrite and nitrate ions. The specific growth rate of *N. europaea* and *N. winogradskyi* showed 1.53 day^{-1} and 0.65 day^{-1} , respectively. After co-cultivation, collected microbes showed reusable ability of ammonification and nitrification completed in 4 days.

These findings could be valuable not only as an artificial ammonification and nitrification model, but also for development of organic hydroponics.

CHAPTER III

Isolation of nitrite-oxidizing bacteria with commensal denitrifying bacteria from enriched nitrifying microbial consortia

Nitrification is two-steps microbial processes performed by two bacteria group. Ammonium ions (NH_4^+) is first oxidized to nitrite ions (NO_2^-) by ammonia-oxidizing bacteria (AOB) and archaea (13, 27, 33, 66). Subsequently, NO_2^- is oxidized to nitrate ions (NO_3^-) by nitrite-oxidizing bacteria (NOB) (1, 9, 25, 58, 67). The extensive studies have been focused on AOB and NOB activity in natural water (7, 8). It is possible to investigate the AOB and NOB population with molecular method by targeting of the ammonium monooxygenase (*amoA*) gene and nitrite oxidoreductase (*nxrA*) gene (42, 51, 54, 57, 61). However, culture of nitrifying bacteria is difficult because of their sensitivity to many external factors such as pH and temperature change (16, 38, 46, 47, 50). Until now, only few AOB and NOB are available. Therefore, it was desired to isolate AOB and NOB for analysis of their characters.

The enrichment method of nitrifying bacteria was developed by using soil microorganisms. Nitrifying microorganisms in soil were enriched by controlling amount of organic fertilizer under aerobic condition (36, 60). In chapter 1, the author revealed that enriched nitrifying microbial consortia from soil achieved stable and accelerated organic nitrogen transformation to nitrate (55). PCR-DGGE analysis revealed that the specific heterotrophic bacteria and autotrophic nitrifying bacteria in each ammonification and nitrification stage were demonstrated. In particular, *Nitrobacter* sp. became dominant in nitrite accumulation stage (55). This result showed the probability to isolate NOB from the enriched nitrifying microbial consortia (ENMC). Therefore, in this chapter, the author addressed to isolate NOB enriched in ENMC.

MATERIALS AND METHODS

Enriched nitrifying microbial consortia preparation

Sanyo-bark compost, prepared from the bark of the tree (Sanyo Chip Kogyo, Yamaguchi, Japan), was used as soil microorganism source. Microorganisms were extracted from Sanyo-bark compost (5 g/L) after it was added to paper bag filters and immersed in 800 mL of distilled water for 24 h (soil soak solution). After 24 h, the paper bags were removed from the soil soak solution, and 0.5 g/L of a fish-based soluble fertilizer (total N 7.3%, total C 21.6%, and C/N ratio: 2.9:1) (Cooperatives Yaizu Fisheries Processing Center, Shizuoka, Japan) was added every day to the solution for 5 days. 1 g/L of oyster shell lime (Urabe-Sangyou, Hiroshima, Japan) were added to provide additional nutrients and minor nutrient.

The nitrogen-ions transition during nitrification was monitored using Ammonium-Test, Nitrite-Test, and Nitrate-Test (Merck, Frankfurt, Germany).

Isolation of nitrite-oxidizing bacteria from ENMC

After the nitrification was finished (NO_2^- was not detected and NO_3^- became constant), the enriched nitrifying microbial consortia (ENMC) were collected by centrifugation at 8,000 rpm (5,800 g) for 10 min. The ENMC was spread on Boon's (4) medium plate (2.0, 5.0, 8.0 and 10.0 g NaNO_2 , 3.2 $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.272 g KH_2PO_4 , 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 20 g gellan gum per liter of distilled water, pH 7.6). After incubation at 28°C for 2 weeks, the colonies were picked up and sub-cultured on Boon's plates. The nitrite-oxidizing activity of the picked up colonies were monitored in Boon's liquid medium using Nitrite-Test and Nitrate-Test.

Analysis of the nitrite-oxidizing bacteria

DNA extraction

Total genomic DNA extraction of isolated No. 21 was performed using ISOIL for Beads Beating (NIPPON GENE, Tokyo, Japan). The extracted DNA was used for polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis

and sequence analysis.

PCR reactions and DGGE analysis

PCR was performed using a T-3000 Thermo cycler (Biometra, Göttingen, Germany). Primer pairs used to analyze communities of NOB was F2842 F1 nxrA GC and F2843 R2 nxrA (69) (Table 3-1).

PCR amplification of NOB was performed in a reaction mixture (50 μ L) containing 1 μ L of genomic DNA, 1 μ L of Ex taq HS (TaKaRa-Bio, Shiga, Japan), 25 μ L of 2 $\times$ PCR buffer, 2 mM of each dNTP, and 0.25 μ M of primers. The PCR program was 50 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s.

The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were analyzed by DGGE using the DCode System (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions (13). For analysis of NOB, 6% of polyacrylamide gels with denaturing solution gradients of 50%-70% (100% denaturing solution contained 40% formamide and 7.0 M urea) were prepared. Electrophoresis was performed with a constant voltage of 50 V at 58°C for 18 h. DGGE gels were stained with Ez Stain Silver (ATTO, Tokyo, Japan) and dried using the Gel-Dry Kit (TEFCO, Tokyo, Japan). Marker for DGGE analyzes of NOB were DGGE Marker III (Nippon Gene, Toyama, Japan).

Identification of isolated colony by 16S rDNA sequence analysis

For 16S rDNA sequencing, the primers 27F and 1525R (Table 3-1) were used to analyze. PCR amplification was carried out by using KOD FX Neo (TOYOBO, Osaka, Japan). The PCR products were electrophoresed on 2% agarose gel with ethidium bromide staining and about 1,500 bp DNA band was cut off. The DNA was recovered by using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The amplified products were cloned in pUC118 using the Mighty Cloning Reagent set (Blunt End) (Takara, Shiga, Japan). The competent cell containing plasmid DNA was purified by using QIAprep Spin Miniprep Kit (QIAGEN, Holland).

PCR amplification for sequence analysis was carried out in a total 15 μ L containing 195 ng of purified plasmid DNA, 3 μ L DTCS mix and 1 μ L 0.25 μ M of primer

(Table 3-1). The PCR program was 40 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min. PCR products were mixed with 2 µl 3 M sodium acetate, 2 µl 100 mM EDTA and 1 µl glycogen. Then, 60 µl 100 % ethanol was added to the mixture and centrifuged at 15,000 rpm for 15 min. The supernatants were removed and the pellets were added with 200 µl 70% ethanol and centrifuged at 15,000 rpm for 3 min. This step was performed twice. The supernatants were removed and the pellets was dried at room temperature (25°C). Then, the dried samples were dissolved in 30 µl Sample Loading Solution.

The DNA sequences were read by GenomeLab™ CEQ 8000 (Beckman Coulter, USA). The sequence data was analyzed by ATGC sequence assembly software Ver. 6 (Genetix, Tokyo, Japan) and identified using BLAST.

Microscopic observation

Microscopic observation of isolated nitrite-oxidizing bacteria was examined by using LEICA Stereo Microscope M205C (Wetzlar, Germany).

Table 3-1 Primer sets for PCR-DGGE analysis and sequence analysis

Primer	Nucleotide sequence
F2842 F1 nxrA GC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCAGACCGACGTGTGCGAAAG-3'
F2843 R2 nxrA	5'-TCCACAAGGAACGGAAGGTC-3'
27F	5'-AGAGTTTGATCMTGGCTCAG -3'
1525R	5'-AAGGAGGTGWTCCARCC -3'
M13F	5'- CGCCAGGGTTTTCCCAGTCACGAC-3'
357F	5'- CTCCTACGGGAGGCAGCAG -3'
533F	5'- GTGCCAGCAGCCGCGGTAA -3'
787F	5'- ATTAGATACCCNNGGTAG -3'
984F	5'- AACGCGAAGAACCTTAC -3'
1378R	5'- CGGTGTGTACAAGGCCCGGGAACG -3'
1492R	5'- GNTACCTTGTTACGACTT -3'
M13R	5'- CACAGGAAACAGCAGCTATGACCATGATT -3'

RESULTS

Isolated nitrite-oxidizing bacteria

On Boon's plate containing 2 g/L of NaNO_2 , 138 colonies were detected. These colonies were investigated for nitrite-oxidizing activities by culturing in Boon's liquid medium. However, the random checking of these colonies did not show nitrite oxidizing activity. On Boon's plate containing 5 g/L of NaNO_2 , 22 colonies were detected. The nitrite-oxidizing activity was observed in the isolated colony No.21. Nitrite was oxidized at day 8 of cultivation and all nitrite was converted to nitrate at day 13 of cultivation (Fig. 3-1 A). The nitrite-oxidizing activity of No. 21 was as same as that of type strain *Nitrobacter winogradskyi* NBRC 14297 which oxidized all nitrite to nitrate at day 10 of cultivation (Fig. 3-1 B). On the other hand, no colony was observed on Boon's plate containing 8 g/L and 10 g/L of NaNO_2 .

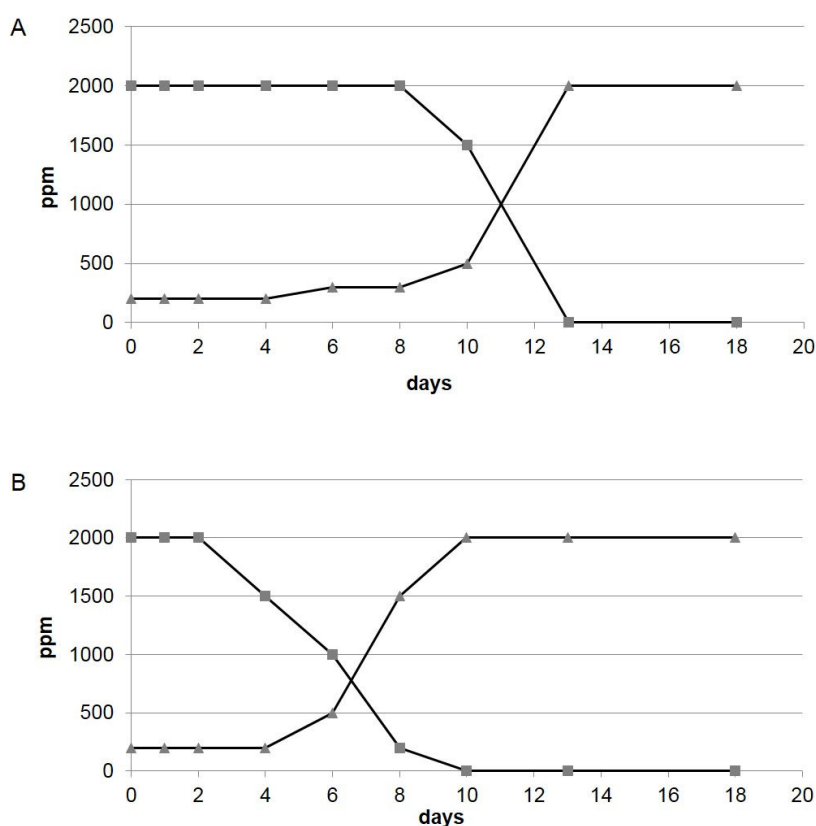


Fig. 3-1 Nitrite-oxidizing activity by the isolated No. 21 (A) and *Nitrobacter winogradskyi* NBRC 14297 (B) ■, NO_2^- ; ▲, NO_3^-

Identification of isolated nitrite-oxidizing bacteria

The 16S rDNA gene sequence analysis based on BLAST database demonstrated that isolated No. 21 containing two strains, *Nitrobacter* sp. and *Alicyclophilus* sp. In order to separate this two strains, the bacteria pair was cultivated in Boon's liquid medium and spread on Boon's plate containing 2 g/L, 5 g/L, and 8 g/L NaNO₂. The various form of colonies on the different concentration of NaNO₂ of Boon's plates were observed using LEICA Stereo Microscope M205C (Fig. 3-2).

The colonies A-E were analyzed of *nxrA* gene by PCR analysis and were identified by 16S rDNA sequence analysis. PCR analysis showed that colonies A and D contained *nxrA* gene. The sequence analysis revealed that the colonies consisted of *Nitrobacter* sp. and *Alicyclophilus* sp. On the other hand, colonies B, C, and E did not contain *nxrA* gene. The sequence analysis revealed that the colonies consisted of *Alicyclophilus* sp.

The isolated nitrite-oxidizing bacteria existing with culturable bacteria was further analyzed by PCR-DGGE using *nxrA* gene for *Nitrobacter*. The observed band revealed that the *Nitrobacter* strain found in the bacterial pair is one of the main nitrite-oxidizing bacteria in the enriched nitrifying microbial consortia (ENMC) (Fig. 3-3). However, the observed band of the bacteria pair was clearly different from the type strain, *N. winogradskyi* NBRC 14297. This result showed that the *Nitrobacter* strain found in the bacterial pair was not *N. winogradskyi* NBRC 14297.

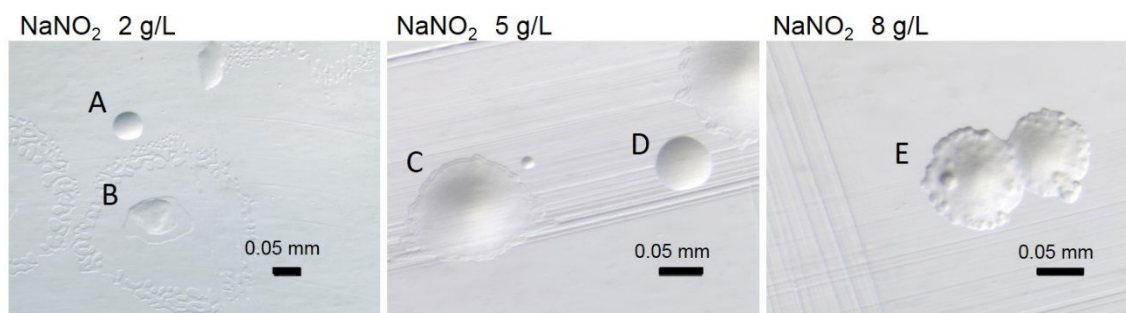


Fig. 3-2 Microscopic observation of isolated No. 21 on Boon's media plates containing 2, 5, and 8 g/L NaNO₂

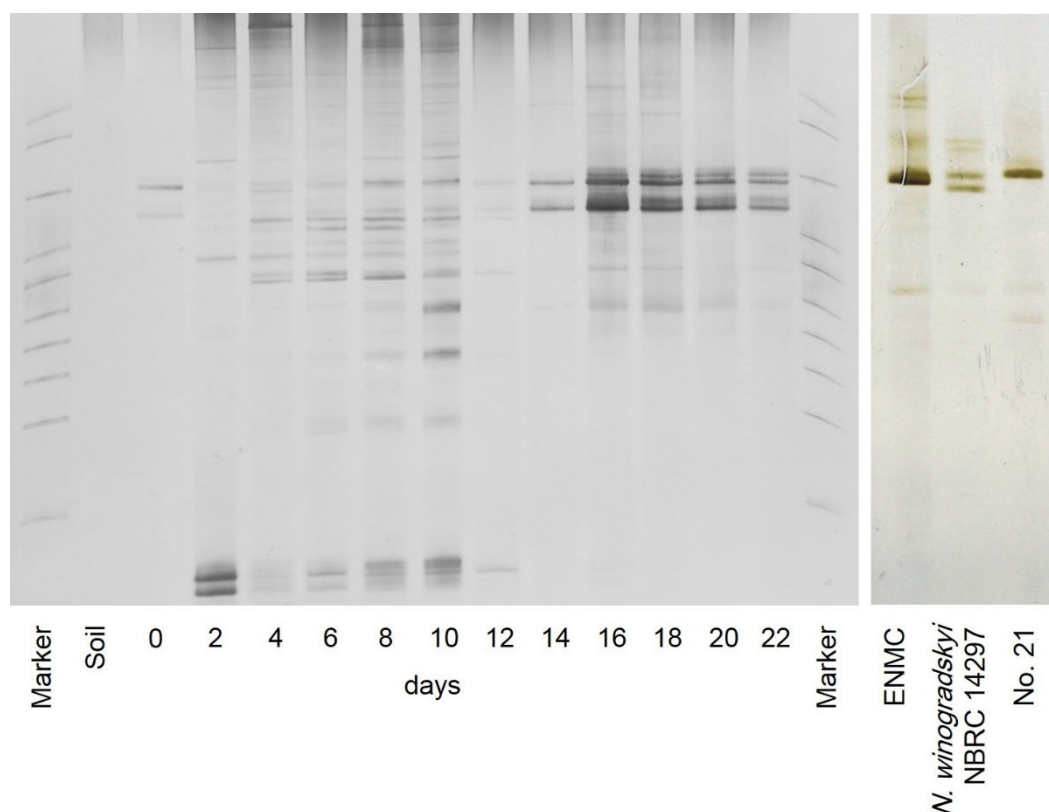


Fig. 3-3 PCR-DGGE analysis of isolated No. 21 using *nxrA* (nitrite oxidoreductase) gene

DISCUSSION

In this study, nitrite-oxidizing bacteria existing with culturable commensal bacteria was isolated from the enriched nitrifying microbial consortia (ENMC). The concentration of NaNO_2 was considered to avoid the contamination of heterotrophic bacteria. Boon's medium containing low concentration of NaNO_2 (2 g/L) led to contamination of heterotrophic bacteria. In contrast, no colony was observed on Boon's medium containing high concentration of NaNO_2 (8 g/L and 10 g/L). The one colony of nitrite-oxidizing bacteria was obtained from Boon's medium containing 5,000 ppm of NaNO_2 .

The isolated bacteria show the various form of colonies on the different concentration of NaNO_2 (Fig. 3-2). The smooth circle colonies (A and E) have *nxrA* gene. However, the sequence analysis revealed that these colonies contained *Nitrobacter* sp.

and *Alicyclophilus* sp. These results indicated that occupancy of *Nitrobacter* sp. was higher than that of *Alicyclophilus* sp. On the other hand, the sequence analysis showed that *Alicyclophilus* sp. was dominant in rough colonies (B, C, and D) in which *nxrA* gene was not detected. These results suggested that *Alicyclophilus* sp. could grow independently. In contrast, *Nitrobacter* sp. could form colony only when it co-existed with culturable *Alicyclophilus* sp. Moreover, the colony observations suggested that ratio of the strain existing in the bacteria pair affected to the colony morphology.

Alicyclophilus is a facultative anaerobe which known as the main denitrifiers that could use nitrate and oxygen as electron acceptors for growth (43, 45, 59). This result revealed that the isolated *Nitrobacter* sp. is co-existing with *Alicyclophilus* sp. However, The symbiosis of these two bacteria still unclear. One of the possible mechanisms is the balance of nitrogen-ions re-cycling. Nitrate is reduced to nitrite by *Alicyclophilus* sp. and nitrite is oxidized to nitrate by *Nitrobacter* sp. The bacteria pair will be useful for understanding the mechanism of the symbiosis.

SUMMARY

A colony-forming nitrite-oxidizing bacteria existing with culturable commensal bacteria (Sample No. 21) was isolated from enriched nitrifying microbial consortia (ENMC) was enriched from Sanyo-bark compost under aeration condition. The isolated nitrite-oxidizing bacteria existing with commensal bacteria showed nitrite-oxidizing activity as same as that of *Nitrobacter winogradskyi* NBRC 14297.

The sequence analysis showed that the bacterial pair consisted of *Nitrobacter* sp. and *Alicyclophilus* sp. PCR-DGGE analysis using the probe for *Nitrobacter* revealed that the *Nitrobacter* strain found in the bacterial pair is one of the main nitrite-oxidizing bacteria in the ENMC.

The isolated *Nitrobacter* sp. existing with culturable *Alicyclophilus* sp. will be useful for understanding the mechanism of the symbiosis.

CHAPTER IV

Analysis of pathogenic bacteria in enriched microbial consortia

In soil culture, organic fertilizers are degraded by soil microorganisms. Subsequently, inorganic nitrogen is oxidized via nitrification process by nitrifying bacteria in two oxidation steps (25, 58, 67). Ammonium ions are first oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and archaea (1, 27, 33, 66). Then, nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB) (9, 67) and this nitrate is absorbed by plants.

In conventional hydroponics, organic fertilizers have phytotoxic effects that lead to poor plant growth (20, 30, 37) and are difficult to use because of the lack of a microbial community to degrade organic compounds to nitrate.

Recently, organic hydroponic system was established. The organic hydroponic consists of two processes. The first is enrichment process of nitrifying microorganisms from soil by controlling amounts of organic nitrogen fertilizers under aerobic condition. The organic nitrogen compounds are gradually degrades to nitrate. The second process is crop cultivation with maintaining the enriched nitrifying microbial consortia by addition of organic fertilizer (60).

The enriched nitrifying microbial consortia (ENMC) have been applied to practical organic hydroponics. In order to spread this novel technology in public, it is necessary to assess the safety of the ENMC.

In this chapter, the author assessed the contamination of pathogenic bacteria in the enriched nitrifying microbial consortia and its ability to suppress the contaminated microorganisms during crop cultivation.

MATERIALS AND METHODS

Enriched nitrifying microbial consortia preparation

The enriched nitrifying microbial consortia preparation was described in the chapter III.

The nitrogen ions transition during nitrification were monitored using Ammonium-Test, Nitrite-Test, Nitrate-Test (Merck, Frankfurt, Germany). When NO_2^- was not detected and NO_3^- became constant (Fig. 4-1), this organic hydroponic solution was enabled to use for crop cultivation.

After the nitrification was finished, the enriched nitrifying microbial consortia (ENMC) were collected by centrifugation at 8,000 rpm (5,800 g) for 10 min. and were used for pathogenic bacteria investigation.

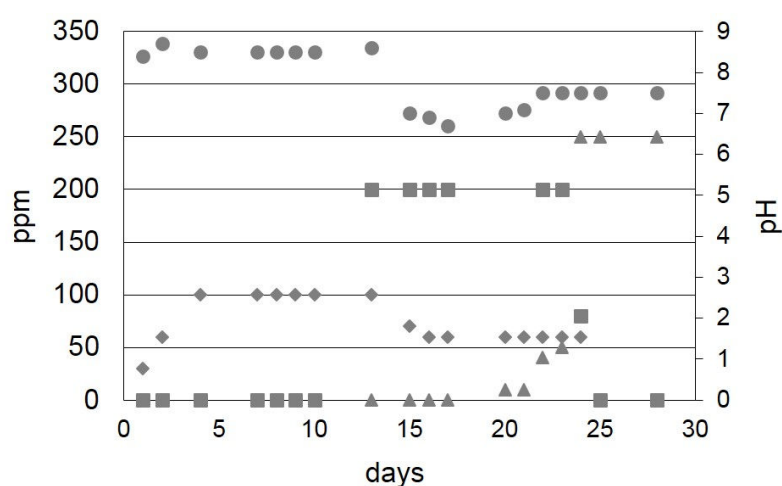


Fig. 4-1 The transition of nitrogen ions and pH performed by the enriched nitrifying microbial consortia ◆, NH_4^+ ; ■, NO_2^- ; ▲, NO_3^- ; ●, pH.

Investigation of pathogenic bacteria

The ENMC collected from organic hydroponic solution was investigated for pathogenic bacteria by serial dilution and plating on various selective media (Table 4-1) specific to *Escherichia coli* (O157, O111, O26) (CHEOM agar, Kanto Chemical, Japan), *Campylobacter* sp. (mCCDA, Eiken Chemical, Japan), *Staphylococcus aureus* (Mannitol Salt agar, As One, Japan), *Salmonella* sp. (MLCB agar, As one, Japan), and *Bacillus cereus* (NGKG agar, As one, Japan).

The incubation conditions and colony color of the pathogenic bacteria were showed in Table 4-1.

Table 4-1 Selective media and culture condition

Target bacteria	Culture medium	Temperature (°C)	Cultivation time (h)	Colony color
<i>Escherichia coli</i> (O157, O111, O26)	CHROM agar	37	20	Pink or blue
<i>Campylobacter</i> sp.	mCCDA agar	42	48-72	gray-white
<i>Staphylococcus aureus</i>	Mannitol Salt agar	35	45-51	yellow
<i>Salmonella</i> sp.	MLCB agar	35	18-24	black
<i>Bacillus cereus</i>	NGKG agar	30	18-24	white

Investigation of ability of ENMC to suppress contaminated microorganisms

Investigation of the suppression in organic hydroponic solution

The organic hydroponic solution was prepared in the Erlenmeyer flasks containing 50 ml of distilled water with addition of 1 g/L ENMC, 1 g/L fish-based soluble fertilizer (Cooperatives Yaizu Fisheries Processing Center, Shizuoka, Japan), and 1 g/L oyster shell lime (Urabe-Sangyou, Hiroshima, Japan). The organic hydroponic solutions were incubated at 25°C, 120 rpm. After 20 days of incubation, *Escherichia coli* JCM 18467 (rifampicin-resistant strain) 10^4 CFU/mL and *Salmonella enteric* ATCC 53648 (rifampicin-resistant strain) 10^4 CFU/mL were inoculated into the hydroponic solutions.

The transition of *E. coli* cells and *S. enteric* cells were investigated by dilution plating method on LB medium containing rifampicin to avoid other microorganisms. The medium compositions in 1 L of distilled water was 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 20 g agar. After autoclave, 50 mg of rifampicin dissolved in 1 mL of DMSO was added into the medium.

Investigation of the suppression during crop cultivation

The organic hydroponic cultivation was prepared in 200 L of distilled water with addition of 150 g ENMC, 100 g (20 g/day for 5 day) fish-based soluble fertilizer (Cooperatives Yaizu Fisheries Processing Center, Shizuoka, Japan), and 200 g oyster shell lime (Urabe-Sangyou, Hiroshima, Japan). When NO_2^- was not detected, 10 L of organic

hydroponic solution was transferred and to each pot for crops cultivations.

Inorganic hydroponic solution was prepared with inorganic fertilizer (OAT house No.1 1.35 g/L, No.2 0.9 g/L, and No. 5 0.045 g; OAT Agrio Co., Ltd.).

Tomato, Chinese cabbage and Boston lettuce were used for inoculation test. At day 6 after transplanting into the hydroponic solutions, each of seedling was inoculated with 10^4 CFU/mL of *E. coli* JCM 18467 (rifampicin-resistant strain). The transition of *E. coli* cells was investigated by dilution plating method on LB medium containing rifampicin to avoid other microorganisms. The medium compositions in 1 L of distilled water was 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 20 g agar. After autoclave, 50 mg of rifampicin dissolved in 1 mL of DMSO was added into the medium.

RESULTS

The pathogenic bacteria in ENMC

Pathogenic bacteria investigation in ENMC as well as in soil (Sanyo-bark compost) showed that no colony of *E. coli* (O157, O111, O26), *Campylobacter* sp. and *Salmonella* sp. was detected on selective media plates (Table 4-2). In contrast, *S. aureus* and *B. cereus* were detected in both ENMC and Sanyo-bark compost. However, the number of *S. aureus* and *B. cereus* detected in the ENMC were lower than those detected in Sanyo-bark compost. The colonies of *S. aureus* and *B. cereus* showed yellow and white color on the selective media (mannitol salt agar and NGKG agar), respectively.

This result suggested that the enrichment method suppress a growth of pathogenic bacteria.

Table 4-2 Colonies detection of pathogenic bacteria from Sanyo-bark compost and enriched nitrifying microbial consortia (ENMC)

Pathogenic bacteria	Sanyo-bark compost	ENMC
<i>Escherichia coli</i> (O157,O111,O26)	-	-
<i>Campylobacter</i> sp.	-	-
<i>Staphylococcus aureus</i>	+++	+
<i>Salmonella</i> sp.	-	-
<i>Bacillus cereus</i>	++	+

Ability of ENMC to suppress the growth of contaminated microorganisms in organic hydroponic solution

To investigate the ability of ENMC to suppress contaminated microorganisms in organic hydroponic solution, *Escherichia coli* JCM18467 and *Salmonella enteric* ATCC 53648 were inoculated to hydroponic solution. After 1 day of inoculation, the *E. coli* cells were below the detection limit (<10 CFU/mL) (Fig. 4-2). On the other hand, the *S. enteric* cells showed the decreasing after 1 day of inoculation (1.8×10^3 CFU/mL remaining). The increasing of *S. enteric* cells were observed again at day 3 (up to 7.5×10^3 CFU/mL), however, no *S. enteric* was detected after 4 days of cultivation (Fig. 4-2).

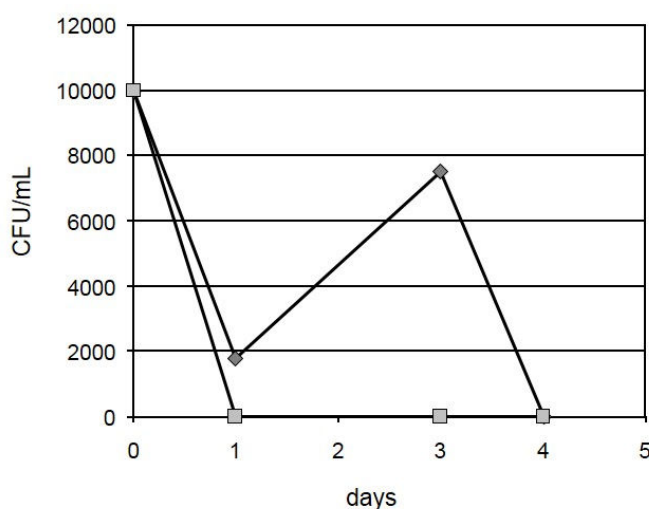


Fig. 4-2 The growth suppression of *Escherichia coli* and *Salmonella enteric* in the organic hydroponic solution, ■ *E. coli*; ◆, *S. enteric*

Ability of ENMC to suppress the growth of contaminated microorganisms during crop cultivation

E. coli JCM 18467 (rifampicin-resistant strain) was inoculated during tomato, Chinese cabbage and Boston lettuce cultivation in both organic and inorganic hydroponic.

Only 2 day, the *E. coli* cells fell below the detection limited (<10 CFU/mL) in all organic hydroponic culture. In contrast, the *E.coli* cells approximately $1-8 \times 10^3$ CFU/mL were remained in the inorganic hydroponic culture. However, the *E.coli* cells were not detected after 6 day of cultivation.

These observation confirmed that the ENMC enable to suppress the growth of contaminated microorganisms.

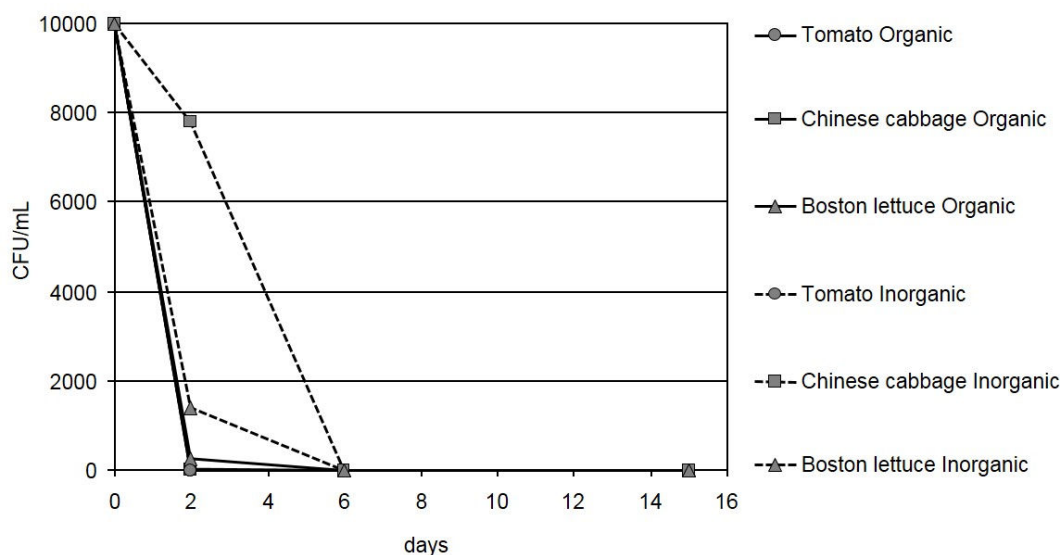


Fig. 4-3 The growth suppression of *Escherichia coli* during crop cultivation in organic and inorganic hydroponic cultivations

DISCUSSION

Organic hydroponics is a sustainable production system which enable efficient vegetable cultivation. Because of the concern about food safety, organic hydroponics have been focused. This study confirmed the safety in terms of pathogenicity of organic hydroponics. The number of pathogenic bacteria detected in the enriched microbial

consortia (ENMC) were lower than those detected in the microorganism source. Moreover, the ENMC showed ability to suppress the growth of contaminated microorganism during cultivation process.

The ENMC achieved a stable and accelerated organic nitrogen transition to nitrate. The organic hydroponic system seem to be strong by keeping the equilibrium of nitrogen compounds transformation. Organic nitrogen compounds were efficiently transformed to nitrate and nitrate was absorbed by plants. The system showed the probability that no nitrogen-ions remained in the organic hydroponic solution, resulted in dramatic decrease of inoculated *Escherichia coli* and *Salmonella enteric* (Fig.4-2 and 4-3). These results confirmed the safety in terms of pathogenicity of organic hydroponics using enriched nitrifying microbial consortia.

SUMMARY

The enriched nitrifying microbial consortia (ENMC) was assessed the biological safety in terms of pathogenicity. The ENMC was evaluated by dilution plating method using various selective media specific to *Escherichia coli* (O157, O111, O26), *Staphylococcus aureus*, *Campylobacter* sp., *Salmonella* sp. and *Bacillus cereus*. The number of *S. aureus* and *B. cereus* detected in the ENMC were lower than those detected in Sanyo-bark compost and the others were not detected.

The ability of ENMC to suppress the contaminated microorganisms was investigated during crop cultivation process. *Escherichia coli* (10^4 CFU/mL) was inoculated into the organic and inorganic hydroponic during tomato, Chinese cabbage, and lettuce cultivation. Only 2 day, the *E. coli* cells were disappeared from organic hydroponic. In contrast, the *E.coli* still remained in the inorganic hydroponic.

This results confirmed the safety in terms of pathogenicity of organic hydroponics using enriched nitrifying microbial consortia.

CONCLUSIONS

CHAPTER I

Nitrifying microbial consortia were enriched from bark compost in a water system by regulating the amounts of organic nitrogen compounds and by controlling the aeration conditions with addition of CaCO_3 for maintaining suitable pH. Repeated enrichment showed reproducible mineralization of organic nitrogen via the conversion of ammonium ions (NH_4^+) and nitrite ions (NO_2^-) into nitrate ions (NO_3^-).

The change in microbial composition during the enrichment was investigated by PCR-DGGE analysis with a focus on prokaryote, ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and eukaryote cell types. The microbial transition had a simple profile and showed clear relation to nitrogen ions transition. *Nitrosomonas* and *Nitrobacter* were mainly detected during NH_4^+ and NO_2^- oxidation, respectively.

These results revealing representative microorganisms acting in each ammonification and nitrification stages will be valuable for development of artificial simple microbial consortia for organic hydroponics consisted of identified heterotrophs and autotrophic nitrifying bacteria.

CHAPTER II

A microbial co-culture which consisted of identified heterotrophic bacteria and *Nitrosomonas europaea* NBRC 14298 (ATCC 19718) and *Nitrobacter winogradskyi* NBRC 14297 (ATCC 14123) was constructed for ammonification and nitrification of organic nitrogen under aerobic condition. The author evaluated fifty-four heterotrophic bacteria of denitrification activity in a medium contained high nitrite concentration. The strains which showed nitrite-oxidizing activity without denitrification activity were selected as candidates for co-cultivation.

The co-culture of *Bacillus badius* NBRC 15713 with nitrifying bacteria performed ammonification and nitrification effectively under following conditions; pH range 7-10, various kind of organic nitrogen (derived from fish, soybean, and milk), C/N ratios range 10 to 50, and organic nitrogen compound concentration below 0.2%. When the co-culture was performed in the medium containing Polypepton NF (Total-N 120 mg, C/N ratio 10), the efficiency of inorganic nitrogen recovery rate was up to 47% at 28°C

for 11 day. The population transition of nitrifying bacteria by RT-PCR showed clear relation to the concentration transition of nitrite and nitrate ions. The specific growth rate of *N. europaea* and *N. winogradskyi* showed 1.53 day⁻¹ and 0.65 day⁻¹, respectively. After co-cultivation, collected microbes showed reusable ability of ammonification and nitrification completed in 4 days.

These findings could be valuable not only as an artificial ammonification and nitrification model, but also for development of organic hydroponics.

CHAPTER III

A colony-forming nitrite-oxidizing bacteria existing with culturable commensal bacteria (Sample No. 21) was isolated from enriched nitrifying microbial consortia (ENMC) was enriched from Sanyo-bark compost under aeration condition. The isolated nitrite-oxidizing bacteria existing with commensal bacteria showed nitrite-oxidizing activity as same as that of *Nitrobacter winogradskyi* NBRC 14297.

The sequence analysis showed that the bacterial pair consisted of *Nitrobacter* sp. and *Alicyclophilus* sp. PCR-DGGE analysis using the probe for *Nitrobacter* revealed that the *Nitrobacter* strain found in the bacterial pair is one of the main nitrite-oxidizing bacteria in the ENMC.

The isolated *Nitrobacter* sp. existing with culturable *Alicyclophilus* sp. will be useful for understanding the mechanism of the symbiosis.

CHAPTER IV

The enriched nitrifying microbial consortia (ENMC) was assessed the biological safety in terms of pathogenicity. The ENMC was evaluated by dilution plating method using various selective media specific to *Escherichia coli* (O157, O111, O26), *Staphylococcus aureus*, *Campylobacter* sp., *Salmonella* sp. and *Bacillus cereus*. The number of *S. aureus* and *B. cereus* detected in the ENMC were lower than those detected in Sanyo-bark compost and the others were not detected.

The ability of ENMC to suppress the contaminated microorganisms was investigated during crop cultivation process. *Escherichia coli* (10⁴ CFU/mL) was inoculated into the organic and inorganic hydroponic during tomato, Chinese cabbage,

CONCLUSIONS

and lettuce cultivation. Only 2 day, the *E. coli* cells were disappeared from organic hydroponic. In contrast, the *E.coli* still remained in the inorganic hydroponic.

This results confirmed the safety in terms of pathogenicity of organic hydroponics using enriched nitrifying microbial consortia.

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