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Acclimation of magnetic activated sludge with 1,4-dioxane and analysis of bacterial flora in the sludge

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Abstract

Isolation of pollutant-degrading bacteria is important in bioaugmentation, one of the methods for biological degradation of environmental contaminants. We focused on the magnetic activated sludge (MAS) process as a culture method that efficiently concentrates degrading bacteria, and cultured activated sludge with 1,4-dioxane as a model pollutant. After 860 days of operation, MLVSS, which indicates the amount of sludge, increased from 390 mg/L to 10,000 mg/L, and the removal rate of organic matter including 1,4-dioxane, tetrahydrofuran, and glucose in the artificial wastewater reached up to 97%. Based on these results, the MAS process was successfully used to acclimate activated sludge with 1,4-dioxane. Bacterial flora analysis in the MAS showed that bacteria of the genus *Pseudonocardia*, already reported as 1,4-dioxane degrading bacteria, play an important role in the degradation of this pollutant. The MAS process is a suitable culture method for acclimation of environmental pollutants, and the findings indicate that it can be used as an enrichment unit for pollutant-degrading bacteria.

Keywords: magnetic activated sludge, 1,4-dioxane, tetrahydrofuran, magnetic separation, *Pseudonocardia* sp

1. INTRODUCTION

The technology to biologically remove environmental pollutants is called bioremediation. Bioremediation can be broadly classified into two categories: biostimulation and bioaugmentation. Biostimulation is a method to promote degradation of contaminants by supplying oxygen and nutrients to the contaminated soil and allowing indigenous microorganisms to grow. Bioaugmentation is a method of introducing bacteria that have been proven to biodegrade contaminants into contaminated soil from outside. Pollutant-degrading bacteria are used that have been isolated at the contaminated site or elsewhere. Therefore, it is short-term and more effective than biostimulation. Biological treatment methods are gaining attention as environmentally favorable treatment methods.

Microorganisms that degrade contaminants are essential for bioaugmentation. An efficient way to obtain such microorganisms is to culture activated sludge or contaminated soil as a seed and isolate microorganism that degrade contaminants from the sludge. Therefore, we propose the MAS process as an efficient culture method to acclimate the degrading microorganisms. A common process in biological treatment methods is the standard activated sludge process. Activated sludge is a suspended organic matter containing aerobic microorganisms and suspended solids, and is a method used frequently in sewage treatment plants around the world, including Japan. On the other hand, the Magnetic Activated Sludge (MAS) process is a variation of biological treatment in which magnetite is added to standard activated sludge to prepare a magnetically activated sludge [10-12]. MAS uses magnetic separation of solids and liquids, enabling operation while maintaining a higher sludge concentration (about 10000 mg/L) than standard activated sludge. With these characteristics, the MAS method is expected to be a new sewage treatment method that can solve the problems of conventional activated sludge methods, such as treatment performance, bulking, and generation of excess sludge. Accordingly, we propose the MAS process as an efficient culture method to acclimate degrading microorganisms.

1,4-Dioxane is an organic compound classified as an ether and is used as a synthetic solvent for organic reactions in the chemical and textile industries [1]. When released into the soil, 1,4-dioxane is highly permeable and can reach the subsurface, potentially causing groundwater contamination. It is also a possible carcinogenic compound to animals [1]. In Japan, 1,4-dioxane is a new substance added as an environmental water quality standard in 2009, and it is very important to accumulate more findings on the biodegradation of 1,4-dioxane. Therefore, we selected 1,4-dioxane as a model pollutant.

Recent studies have reported experimental evidence that bacteria of the genera *Pseudonocardia*, *Rhodococcus*, *Afipia*, and *Mycobacterium* are involved in the degradation of 1,4-dioxane [2-8]. Two biodegradation pathways for 1,4-dioxane are believed to exist: degradation using a single carbon and energy source and degradation by co-metabolism. Studies of the co-metabolic degradation of 1,4-dioxane using tetrahydrofuran (THF) as the primary substrate have been reported [9].

In this study, MAS was incubated with 1,4-dioxane as a model contaminant, and the course of incubation and changes in the bacterial flora in the sludge were examined

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to determine its potential as an enrichment unit for 1,4-dioxane degrading bacteria, the basis for bioaugmentation.

2. EXPERIMENTAL PROCEDURES

2.1. Experimental Apparatus

The activated sludge culture was operated in a 5 L acrylic vessel with a magnetic drum and air inlet tubing as shown in Fig. 1. The dimensions of the cultivation vessel are 270 mm × 450 mm × 110 mm. Artificial wastewater flows into the culture tank, where activated sludge (MAS) adsorbed by magnetite and soluble components of decomposed organic and inorganic matter are present. The MAS magnetically attaches to the magnetic drum and is subsequently scraped off with the rotation of the drum and returned to the culture tank. Soluble inorganic matter and soluble residual organic matter are not returned but flow out of the culture tank as effluent.

Artificial wastewater adjusted to pH 7.0 was pumped in at a rate of 5 L/day using a metering pump (MP-3, Tokyo Science Instruments Co., Ltd.), and the hydraulic retention time (HRT) was adjusted to 24 hours. An air pump (Yasunaga Air Pump Co. LP-40A) was used for constant aeration to maintain sufficient dissolved oxygen in the sludge tank. The seed sludge was collected from the return sludge duct of the Kawada Water Reclamation Center in Utsunomiya City (Japan). The composition of the artificial wastewater, excluding carbon sources, is as follows: 27 mg/L $MgSO_47H_2O$, 24 mg/L $CaCl_22H_2O$, 39 mg/L NaCl, 18 mg/L KCl, 21 mg/L KH_2PO_4, 92mg/L $NaHPO_412H_2O$, 18 mg/L Urea.

The percentage of carbon sources added are shown in Fig. 2. 1,4-dioxane, glucose, and tetrahydrofuran (THF) were used as carbon sources. THF began to be supplied on day 56 of incubation. The percentage of carbon source added was varied according to the number of days of incubation, and the amount of glucose added was set to 0 after day 752. Since it is assumed that only a few 1,4-dioxane degrading bacteria exist in activated sludge used to treat domestic wastewater, glucose, a common carbon source available to many kinds of bacteria, was added in the early stages of culture. As acclimation progressed, the concentration of 1,4-dioxane and its co-metabolite, THF, were added to increase the number of 1,4-dioxane-degrading bacteria.

2.2. Analysis

MLSS and MLVSS were measured with reference to Japanese Industrial Standards (JIS) K0102 (2013). Mixed liquor suspended solids (MLSS) is the dry weight concentration of suspended solids in the sludge mixture, and mixed liquor volatile suspended solids (MLVSS) is the weight loss of MLSS after intense heat (600℃). The former is used as an indicator of sludge quantity, while the latter is used as an indicator of the amount of organic matter in suspended solids. Since MAS containing magnetite is used, MLSS includes the weight of magnetite. Therefore, MLVSS was used as an indicator of organic suspended solids (sludge) in this study.

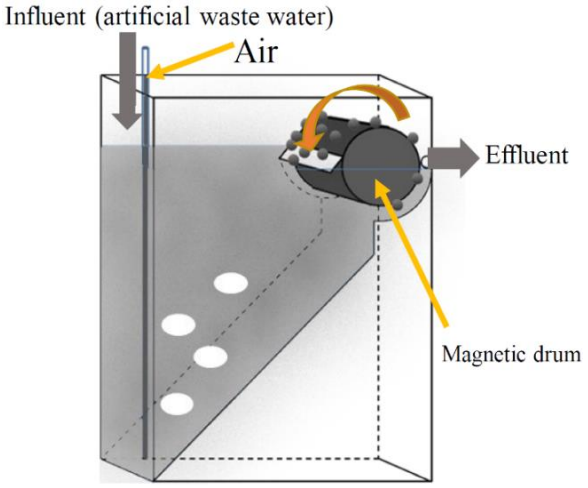

Fig. 1. A schematic diagram of the MAS cultivation vessel.

COD_{Cr} was measured as an indicator of organic pollutants in the water. Potassium dichromate was added to the sample water as an oxidant, and the amount of oxygen required for oxidation was calculated from the amount of oxidant consumed after the reaction. Dissolved oxygen (DO) indicates the amount of oxygen dissolved in the sample water. DO was measured using a portable dissolved oxygen meter (DO-31P, Toa DKK Co., Ltd.) to ensure that sufficient oxygen was supplied.

2.3. 16S rRNA gene amplicon analysis

Since total DNA extracted from sludge is DNA derived from microorganisms constituting the sludge, it is possible to analyze the type and percentage of microorganisms constituting the sludge by decoding the sequences of those DNAs and searching for homology with international databases.

In this study, the partial sequence of a gene called 16S ribosomal DNA, which is possessed by bacteria, was the target of analysis. 500 µL of magnetized sludge was taken and total DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the instruction manual. The extracted DNA was quantified by fluorescence using the QuantiFluor ONE dsDNA system (Promega) and subjected to qualified analysis by using agarose gel electrophoresis. Next generation sequencer (NGS) analysis was performed on 17 DNA samples extracted at 0, 32, 52, 75, 112, 175, 253, 345, 387, 430, 485, 542, 597, 641, 704, 751 and 786 days after the start of incubation.

2.4. PCR amplification of SDIMO alpha-subunit gene

Polymerase chain reaction (PCR) is a technique to amplify specific DNA regions using DNA polymerase, a DNA replication enzyme. Soluble di-iron monooxygenase (SDIMO) is an enzyme presumed to be involved in the initial oxidation of 1,4-dioxane and THF, and this enzyme is an indicator of the presence of bacteria that degrade both substances. Using total DNA extracted from sludge as template DNA, PCR can be used to selectively amplify and detect the SDIMO gene. Using this technique, we attempted to detect bacteria involved in 1,4-dioxane

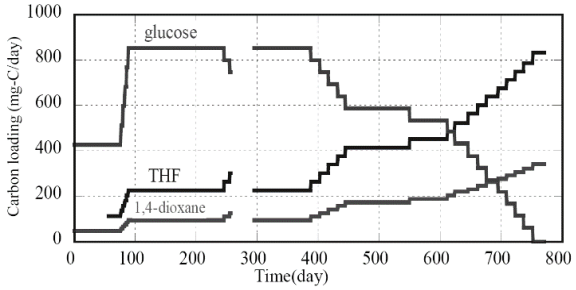

Fig. 2. Incubation period and changes in the supply of the three carbon sources.

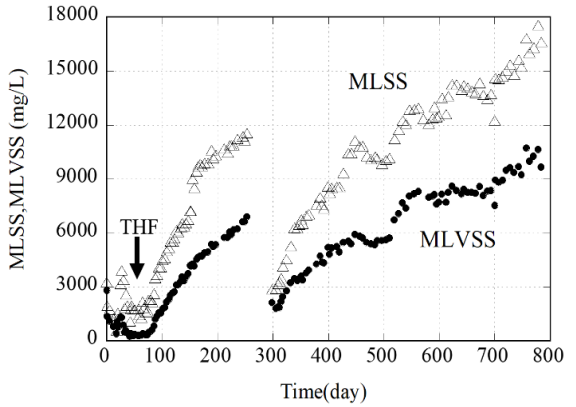

Fig. 3. MLSS and MLVSS variations with culture Vertical black arrows indicate the time at which THF was added.

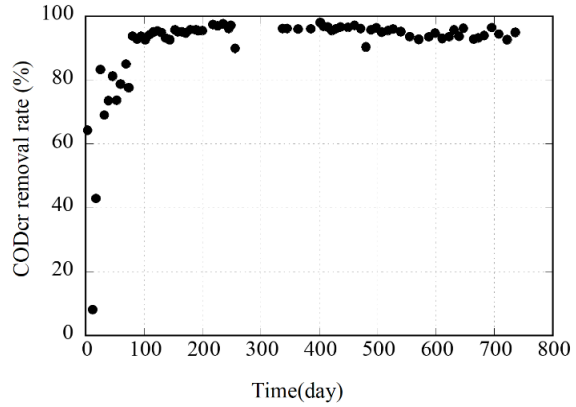

Fig.4. Organic matter removal rate calculated from COD_{Cr} measurements in effluent

degradation in acclimated sludge. Agarose gel electrophoresis was also used to detect DNA amplified by PCR. When negatively charged DNA moves in the agarose gel toward the anode, its mobility is affected by the molecular weight of the DNA. Agarose gels from which PCR products were separated were detected using the DNA-specific fluorescent staining dye Midori Green (Fast Gene).

The SDIMO gene possessed by the sludge-constituting bacteria was detected using PCR. Extracted DNA was used as template and amplified with SDIMO alpha-subunit gene-specific primer sets (NVC57: CAGTCNGAYGAR-KCSCGNCAYAT, NVC66: CCANCCNGGRTAYTTR-TTYTCRAACCA) [13] and PCR enzyme (KOD FX NEO, TOYOBO Co). The PCR reaction consisted of an initial denaturation for 2 minutes before 35 cycles of denaturation for 10 seconds, annealing reaction for 30 seconds, and elongation reaction for 30 seconds.

3. RESULTS AND DISCUSSION

3.1. Behavior of pH, DO, MLSS and MLVSS in culture

Throughout the 752 days of incubation, the effluent pH ranged from 6.6-7.4 and dissolved oxygen (DO) remained approximately above 3 mg/L even after sludge concentrations began to increase. In magnetized activated sludge, MLSS, the dry weight concentration, will include magnetite, while MLVSS is the appropriate indicator for net sludge concentration. At the start of the culture, MLSS and MLVSS were 3248 and 2784 mg/L respectively and artificial wastewater with glucose and 1,4-dioxane as carbon sources was used. MLVSS then decreased to 390 mg/L on day 18 of incubation. This was thought to be due to the death of a group of bacteria that could not degrade or tolerate 1,4-dioxane and the resulting washout from the culture tank. Subsequently, THF was added as a third carbon source starting on day 56 of incubation, and the inflow load was increased stepwise starting on day 77, resulting in a gradual increase in MLVSS and stable sludge production. Although there was a decrease in sludge from day 257 to 294 of incubation due to the temporary interruption of the experiment caused by the pandemic (gray zone in the graph), the sludge stabilized and increased as incubation resumed. The fact that MLVSS remained constant at about 8000 mg/L under constant magnetic powder concentration (Fig.3.).

The small variation in MLVSS from day 546 to day 693 of incubation was considered to be a balance between growth and self-digestion within the magnetized activated sludge. After day 752 of incubation, a temporary decrease in MLVSS was observed as a result of removing glucose from the carbon source of the artificial effluent. This was attributed to the death of bacterial species that could not degrade 1,4-dioxane or THF. However, after 820 days of incubation, the magnetized activated sludge fed with artificial wastewater with only 1,4-dioxane and THF as carbon sources began to increase again, and MLVSS increased to 10540 mg/L by day 867 of incubation. This increase in sludge suggests that a microbial population capable of utilizing 1,4-dioxane or THF is clearly proliferating and present within the magnetized activated sludge.

3.2. Removal of organic matter by MAS process

The organic matter removal rate calculated from the COD_{Cr} measurement results is shown in Fig. 4. Up to day 56 of incubation, the removal rate of COD_{Cr} in the effluent was about 75% at most, resulting in low treated water quality. Since the sludge in the early incubation period was presumed to be dominated by bacteria that were not resistant to the toxicity of 1,4-dioxane, it was thought that autolysis was more significant than growth on glucose during this period. With the addition of THF from day 56 and the increase in carbon source from day 77, the COD_{Cr} removal rate increased to 92~96%, and the high COD_{Cr}

removal rate was maintained as the culture continued. The 1,4-dioxane removal rate, which was estimated assuming that all glucose and THF were degraded by the sludge, gradually increased from about 60% to 80% after the 400th day of incubation, and increased up to 89.6% (823rd day) after the addition of no glucose as a carbon source. Although this study did not directly measure 1,4-dioxane in the effluent, it is clear that the MAS process can acclimate sludge capable of removing most of the high concentration of 1,4-dioxane.

CODcr in the influent was approximately 600 mg/L. On the other hand, CODcr in the effluent was unstable up to 100 days of incubation, but as the incubation continued, CODcr in the effluent became stable in the range of 12.3 mg/L to 59.4 mg/L up to 752 days of incubation. Although we did not directly measure 1,4-dioxane concentrations in the effluent up to 752 days of incubation, direct measurement of 1,4-dioxane at 1235 days of incubation showed good removal at 0.28 mg/L, meeting the Japanese 1,4-dioxane effluent standard of 0.5 mg/L.

3.3. Increase in SDIMO gene-carrying bacteria

Soluble di-iron monooxygenase (SDIMO) is an important enzyme involved in the initial oxidation of THF and 1,4-dioxane in its aerobic degradation [8]. The SDIMO gene was detected to confirm the presence of 1,4-dioxane degrading bacteria in the MAS over the course of the culture. In order to identify SDIMO-possessing bacteria in the MAS acclimated in this study, total DNA extracted from the sludge before and after the addition of THF was used as a template to amplify the SDIMO alpha-subunit using a specific primer set. The partial SDIMO gene DNA fragment detected was approximately 420 bp and is indicated by the black horizontal arrow in Fig. 5. The DNA fragment was not detected before 52 days of incubation, while it was detected after the time when THF was started to be added (57 days). This suggests that the addition of THF may have started to increase the number of bacteria degrading THF and 1,4-dioxane with SDIMO.

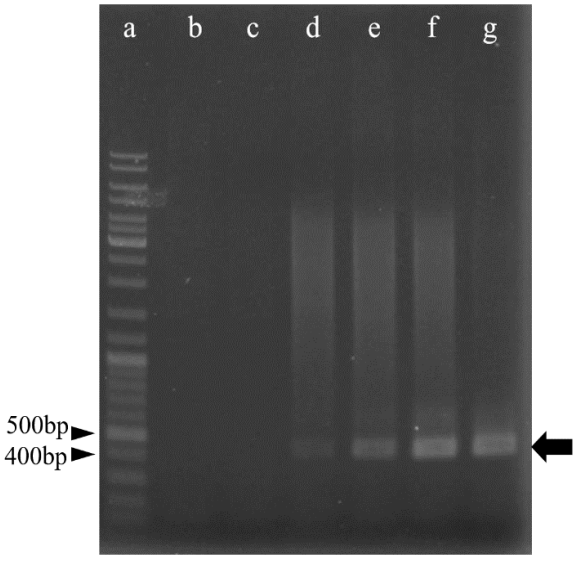

Fig. 5. Electrophoresis image of PCR amplicon targeting SDIMO gene alpha-subunit
a: DNA molecular marker, b: day 32, c: day 52, d: day 58, e: day 63, f: day 66, g: day 75 of incubation.

3.4. Bacterial flora of 1,4-dioxane acclimated sludge

The bacterial flora within the magnetically activated sludge at the phylum (Fig. 6) and genus (TABLE I) levels were analyzed using NGS. At the phylum level, Proteobacteria was the most dominant phylum during all periods of incubation, regardless of variations in the amount or percentage of carbon source added.

Bacteroidetes was the second most dominant phylum. The phylum Actinobacteria, in which most of the bacteria albeit in small proportions, during all periods of culture.

Of the bacterial genera whose relative abundance exceeded 3%, even temporarily during the incubation period, 23 genera were identified (TABLE I). At the genus level, *Ideonella* sp., *Pseudomonas* sp. and *Sphaerotilus* sp. were present in the early stages of culture, even if only temporarily, at a percentage greater than 10%. These bacteria are commonly present in standard activated sludge and were thought to be dominant due to their high metabolic rates, especially for organic matter such as glucose.

Pseudonocardia spp. were not detected until day 52 of culture, but continued to be observed as soon as THF addition began, and became the most dominant (4.54%) of the bacterial species identified by day 786 of culture, when only 1,4-dioxane and THF carbon sources were supplied. Since *Pseudonocardia* spp. are classified under phylum Actinobacteria, the increase in phylum Actinobacteria at 786 days of incubation after glucose addition was stopped in Fig. 6 is probably due to an increase in *Pseudonocardia* spp. *Pseudonocardia* spp. have been reported to include *P. acaciae* and *P. asaccharolytica*, which co-metabolize 1,4-dioxane using THF as a primary substrate [3, 4]. 16S rRNA gene amplicon analysis suggested that *Pseudonocardia* spp. play an important role in the degradation of 1,4-dioxane in the magnetically activated sludge cultured in this study, which is dependent on the reaction of co-metabolism with THF as the primary substrate. Although no examples of 1,4-dioxane degradation of *Acidibacter* sp., *Ferruginibacter* sp., *Chthoniobacter* sp., *Haliangium* sp. and *Piscinibacter* sp. have been reported, these bacteria may have the potential to degrade 1,4-dioxane, as their abundance increased in the later stages of culture.

3.5. Usefulness of the MAS process in acclimation and enrichment of degrading bacteria

In the standard activated sludge method, a common wastewater treatment method, suspended solids (sludge) that form aggregates are separated by gravity, which takes time and operates at a relatively low sludge concentration (about 1000 mg/L). On the other hand, the MAS process can operate at a higher MLSS (more than 10 times higher than the MLSS used in standard activated sludge) because of its strong solid-liquid separation using magnetic force. In culturing at high microbial concentrations, microorganisms that can efficiently take up the supplied carbon source and convert it into metabolic energy will be able to survive to an advantage. As a result, microorganisms with low degradation efficiency would be

TABLE I
GENUS OF BACTERIA THAT BECAME DOMINANT DURING THE INCUBATION PERIOD

Genus	0	32	52	75	112	175	253	345	387	430	485	542	597	641	704	751	786
Acidibacter	0.21	0.01	0	0	0	0.06	0.15	0.10	0.21	0.83	1.95	4.53	4.38	5.12	6.29	5.82	2.60
Ampullimonas	0	0	0.50	6.11	0.71	0.09	0	0	0	0	0	0	0	0	0	0	0
Arenimonas	0	0.07	0.52	0.39	0.12	0.19	0.38	3.11	0.83	0.84	0.99	0.69	0.27	0.23	0.12	0.05	0.06
Chthoniobacter	0	0	0.21	0.51	0.09	0.34	1.16	0.18	0.49	0.66	2.34	3.97	5.26	6.42	8.36	5.64	1.10
Dechloromonas	0.35	0.35	0.59	5.57	0.03	0.04	0.03	0	0	0	0	0.09	0	0.03	0.03	0	0
Dokdonella	0.92	0.02	0	0	0	0.19	0.15	1.48	1.41	4.12	1.32	0.27	0.05	0.07	0.05	0.01	0.01
Ferruginibacter	0.91	0.50	0.06	2.34	4.09	1.09	0.25	0.76	0.30	0.44	3.59	8.11	17.7	10.6	4.20	2.43	2.66
Flavobacterium	0.83	2.23	4.87	0.50	0.25	1.03	0.06	0.02	0.05	0.06	0.06	0.21	0.11	0.08	0.01	0	0.02
Haliangium	5.69	0.03	0.03	0.31	0.74	0.74	0.98	0.51	2.47	1.79	5.20	1.88	4.01	1.09	0.69	1.04	3.20
Haliscomenobacter	0.04	0.30	0.05	0.07	0.03	0.05	0.10	5.25	5.55	2.95	0.42	1.31	0.07	0.05	0.01	0	0
Hyphomicrobium	0.08	0.02	0.03	0.09	0.77	3.02	2.77	3.16	3.59	2.18	2.36	1.73	1.21	0.89	0.46	0.12	0.03
Ideonella	0.19	1.02	19.6	0.21	0.24	0.09	0	0.12	0.10	0.10	0.07	0.24	0.02	0.03	0.05	0	0.01
Kouleothrix	2.49	1.19	0	0	0.05	8.36	2.74	3.77	8.30	4.27	7.85	5.99	3.82	1.27	0.64	0.45	1.50
Methylibium	0	0	0.15	1.26	1.00	1.83	6.56	1.90	3.87	3.28	1.75	0.58	0.16	0.08	0	0	0
Methyloversatilis	0.05	0.03	2.04	9.89	1.17	0.07	0	0	0	0.02	0	0	0	0	0	0	0
Nitrospira	4.11	0.11	0.09	0.39	0.30	0.25	0.67	1.20	1.58	2.06	3.32	1.40	2.88	2.55	1.72	1.42	2.52
Piscinibacter	0.47	0.09	0	0	0.08	2.65	0.06	0	0	0	0	0.16	0.32	0.57	1.64	3.61	1.78
Pseudomonas	0	1.80	12.3	3.32	0.36	0.11	0.07	0.03	0.03	0.07	0.10	0.24	0	0.07	0.12	0.10	0
Pseudonocardia	0	0	0.	1.73	0.79	0.45	0.51	0.19	0.35	0.39	0.39	0.08	0.27	0.19	0.97	1.16	4.54
Raoultella	0.03	0.37	0.46	0.04	4.85	0.04	0	0.10	0.23	0.10	0	0	0.03	0	0	0	0
Rhodobacter	0.37	1.19	3.66	1.57	0.03	0.01	0	0.02	0.07	0.08	0.02	0.02	0	0.01	0.03	0.01	0.02
Sphaerotilus	0.59	37.4	1.08	0.47	21.4	6.41	1.40	6.57	1.03	0.30	0.07	1.03	0.10	0.01	0	0	0
Vogesella	6.28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

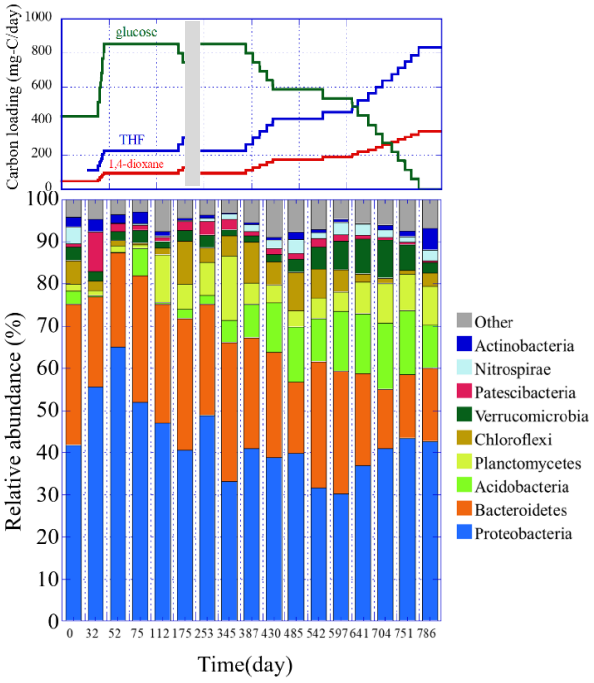

Fig. 6. Relative abundance of bacteria at the phylum level in MAS

reduced and degrading bacteria would be more easily concentrated in the tank. Furthermore, the MAS process has the potential to retain bacteria with a wide range of growth rates, since even low growth rate bacteria that adsorb to magnetite are efficiently returned to and retained in the tank. In the MAS process, microorganisms that are not adsorbed to magnetite are discharged from the tank, but our data to date indicate that filamentous bacteria and fungi are not easily adsorbed to magnetite and are discharged out of the tank. The MAS process retains a wide variety of microorganisms other than those with these properties, and by keeping them in the tank at high concentrations over a long period of time, it is thought that bacteria capable of degrading specific organic matter can be concentrated. We propose the use of the MAS process as an "enrichment unit" that can efficiently cultivate bacteria with specific degrading activity using magnetic separation, and believe that it can be used to isolate contaminant-degrading bacteria for use in bioaugmentation.

For future studies, it would be very important to conduct 1,4-dioxane acclimation using the standard activated sludge method and compare acclimation efficiencies in order to more clearly demonstrate the advantages of the MAS process.

4. CONCLUSION

The MAS method was operated using urban sewage activated sludge as seed sludge and 1,4-dioxane as the main carbon source. As a result, the MAS method observed a good increase in sludge and was able to degrade most of the added 1,4-dioxane. Bacterial flora analysis indicated that *Pseudonocardia* sp. contributed to the degradation of 1,4-dioxane by co-metabolism with THF as the primary substrate, but also suggested the existence of as yet unknown 1,4-dioxane degrading bacteria. In the MAS method, the bacteria are retained in the culture tank while adsorbing on the magnetic powder, which is thought to have enabled efficient acclimation because it can continue

to retain bacterial groups with small growth rates, such as *Pseudonocardia* spp. These results indicate that the MAS method can be used for efficient acclimation of 1,4-dioxane degraded sludge, and that in the future it can be used for bioaugmentation, since it is possible to isolate degrading bacteria from the acclimated sludge.

ACKNOWLEDGEMENT

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