Degradation Kinetics of Geosmin by Biofilm with Organic Carbon as a Substrate

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Abstract

Geosmin is a nuisance secondary metabolism compound by some actinomycete and filamentous cyanobacteria. In waterworks, geosmin is a contaminant, which is resistant to conventional water treatment such as coagulation and sedimentation. There is some information about geosmin degradation in the presence of various organic matters. This study examined influence of glucose, sodium acetate and acetic acid on geosmin degradation by biofilm. The results revealed that presence of glucose and sodium acetate enhanced geosmin biodegradation. On the other hand, inhibition effect on geosmin biodegradation occurred from second day of acetic acid condition. The 16S rDNA copy number did not change dramatically during the experiment in organic carbon additional condition, but ATP concentration increased in glucose condition at 2nd day. This result might be showed that geosmin biodegradation was co-metabolism in the presence of glucose or sodium acetate, which played as a role of secondary substrate.

Keywords: geosmin; biodegradation; biofilm; NOM; stimulation

INTRODUCTION

Geosmin is one of a few chemicals that are responsible for the characteristic earthy smell of soil¹⁾. Geosmin is produced as secondary metabolites by members of certain groups of benthic and pelagic aquatic microorganisms (actinomycetes bacteria (*Streptomyces* spp.) and cyanobacteria) found in source water such as lakes, reservoirs and running water²⁾, and it is a highly-odoriferous, earthy-smelling compound. In waterworks, geosmin is a contaminant, which is resistant to conventional water treatment such as coagulation and sedimentation²⁻⁴⁾.

The odour detection threshold of human has been reported at concentration as low as 1.3 ng l⁻¹ for geosmin^{5),6)}. The presence of taste and odor in potable water supplies is an increasingly frequent problem^{7),8)}. To date, efficient geosmin treatment methods include

adsorption, oxidization by ozone. Although geosmin can be removed effectively by the mentioned techniques, these advanced treatment processes such as ozone oxidation is high cost, and produce hazardous oxidation byproducts when the water containing natural organic matter (NOM)⁹⁾. NOM would also inhibit the power activated carbon (PAC) adsorption efficiency by competing adsorption sites with geosmin, although PAC adsorption is a common and efficient method for geosmin removal in water treatment¹⁰⁻¹²⁾. Therefore, more efficient and practical method for geosmin removal is required.

Geosmin biodegradation by biofilm is proposed as a major pathway for natural elimination of geosmin²⁾, and biofilm has recently been recognized as an efficient and low-cost way to degrade geosmin, and have been used across USA¹³⁾, Australia⁴⁾ and Europe¹⁴⁾.

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Currently, studies regarding the biodegradation mechanism of geosmin are limited. Westerhoff et al.¹⁵⁾ conducted batch culture experiments of geosmin biodegradation using lake water, and they imitated geosmin biodegradation as a pseudo-zero-order reaction. In contrast, Rittmann et al.¹⁶⁾ determined geosmin biodegradation in natural water to be a second-order reaction, due to the presence of NOM which was present at much higher concentration than geosmin (mg l^{-1} , compared with ng l^{-1}). Ho et al.¹⁷⁾ reported that geosmin biodegradation was a pseudo-zero-order reaction. Saito et al.¹⁸⁾ identified four possible biodegradation products of geosmin, two of which were identified as 1,4a-dimethyl-2,3,4,4a,5,6,7,8-octahydronaphthalene. The most important result of this study was the addition of ethanol to enhance geosmin biodegradation. This based on co-metabolism concept.

Geosmin biodegradation was influenced by NOM. In order to remove geosmin from water which contain NOM at $\mu\text{g l}^{-1}$, water treatment plant can adopt biofilter method. But NOM influence for geosmin biodegradation in biofilter step is not clear; especially NOM concentration in water is not high, as $\mu\text{g l}^{-1}$ level. This study aims to identify geosmin biodegradation mechanism by biofilm when different organic carbon source (at $\mu\text{g l}^{-1}$) was coexisted with geosmin. Glucose, sodium acetate and acetic acid is selected and used for organic carbon source, and naturally-originated biofilm from a water treatment plant as a inocula, and the change of bacterial number, activity and community compositions in the biofilm were monitored by 16S rDNA copy number, ATP and DGGE fingerprint.

MATERIALS AND METHODS

Geosmin Geosmin standard was purchased from WAKO pure Chemicals Ltd. Osaka, Japan. The authentic sample of 20 mg geosmin was dissolved in ultra-pure water (Resistivity $18.0 \text{ M}\Omega \cdot \text{cm}$ at 25°C). The stock solution was transferred into brown air-tight glass bottle and stored in the dark at 4°C prior to use.

Batch biodegradation experiments Lake Kasumigaura (Japan) serves as a water source for many cities in Ibaraki prefecture.

The Southern Ibaraki Prefectural Waterworks close by Lake Kasumigaura utilizes the biological treatment facilities. Unit packed with a honeycomb tube is used as carrier for biofilm habitat in these biological treatment facilities. The maximum water supply quantity is $160,000 \text{ m}^3 \text{ day}^{-1}$ and hydraulic time of 2 h. The honeycomb was taken out from water and using sterilized ladle to scraped biofilm attached at the surface of honeycomb tube. Biofilm was collected in sterilized centrifuge tube (15 ml) and stored at 4°C before use.

Geosmin biodegradation experiments were conducted in 1000 ml conical flasks. In each flask, biofilm (fresh, 1.0 g) was added to 500 ml of sterilized mineral salt medium (MSM) including $210 \text{ mg CaCl}_2 \cdot 12\text{H}_2\text{O}$, $130 \text{ mg MgSO}_4 \cdot 7\text{H}_2\text{O}$, $50 \text{ mg Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $10 \text{ mg K}_2\text{HPO}_4$, and $20 \text{ mg (NH}_4)_2\text{SO}_4$ ($\text{pH}=7.6$, per liter of ultra-pure water). The culture was spiked with geosmin to establish an initial concentration 500 ng l^{-1} . As $400 \mu\text{g l}^{-1}$ and $300 \mu\text{g l}^{-1}$ TOC of glucose and sodium acetate (NaAc) was spiked into culture, respectively, which is lower than NOM concentration level (mg l^{-1}) in Lake Kasumigaura. Acetic acid (HAC) was added to form concentration of $0.01\% \text{ V/V}$. HAC was added base on hypothesis that geosmin degradation pathway was same as cyclohexanol, final product of which was unstable diacid, and dosage was reduced because of pH value. The control was conducted which contained sterilized biofilm (autoclaved at 121°C for 20 min) and geosmin to investigate abiotic impact. The subsample of 15 ml was took from each flasks at 0 , 1^{st} , 2^{nd} , 4^{th} , 6^{th} , 8^{th} day for analysis.

Hexane extraction analysis for geosmin and MIB¹⁹⁾ was used to extract the geosmin. Progress of geosmin biodegradation in each flask was monitored by GC/MS analysis in a 2010 plus GC/MS (SHIMADZU, Japan) equipped with a RESTEK Rix-5MS capillary column ($30 \text{ m} \times 0.25 \text{ mm ID} \times 0.25 \mu\text{m}$ fixed phase) with helium as carrier gas. GC-MS condition was list in Table 1. Geosmin biodegradation rate was calculated by linear regression of geosmin concentration of natural logarithm remained in culture as function of time.

TaqMan qPCR analysis For extraction of bacterial DNA, 5 ml of subsample was filtered onto 0.2 µm membrane filter (Nuclepore Track-Etch Membrane, Whatman, UK). The filter was put into the 1.5 ml Eppendorf tube adding 180 µl lysis buffer (50 mM Tris (pH=8.3); 40 mM EDTA; 0.75 M sucrose) to break the cell wall then stored at -80°C before use. DNA extraction was based on phenol-chloroform extraction method²⁰. Initially, adding 5 µl of 50 mg ml⁻¹ lysozyme and incubated for 1 h at 37°C. Then 5 µl of 20 mg ml⁻¹ proteinase K and 20 µl of 10% SDS was added to break the wall of bacteria, and the mixture was incubated for 2 h at 55°C, followed by addition of the same volume PCIA (phenol: chloroform: isoamyl alcohol, 25: 24: 1, pH=8), gently turned tube over for several times followed by centrifugation (15000 g, 30 min, 25°C) and removal of the supernatant for next extraction. PCIA treatment had done for 1-2 times. After

PCIA treatment, the same volume CIA (chloroform: isoamyl alcohol, 24: 1) was added, and then centrifugation (15000 g, 30 min, 25°C), taking the supernatant to the new tube. Then added one-tenth volume of the supernatant of sodium acetate (3 M, pH=7.5) and 2.5 times volume of supernatant of ethanol (100%) to the tube, freezed at -30°C over night. Centrifuging (15000 g, 30 min, 4°C) and discarding the supernatant and using 70% ethanol 1 ml to wash DNA and then removing all ethanol and dry up. Then DNA dissolved in TE buffer (pH=8.0) stored at 4°C for use.

The bacterial number was quantified by quantitative polymerase chain reaction (qPCR) using BACT1369F/PROK1492R primer set and TM1389BACT2 probe²¹) (Table 2). DNA standard curve for total bacterial number was prepared from a serial dilution of purified 1465 bp bacterial 16S rDNA fragment, from a strain *Sphingomonas* sp.

Table 1 GC-MS condition for geosmin analysis

Gas Chromatography (GC) condition	
Column	Rix-5MS (RESTEK), 30 m × 0.25 mm ID × 0.25 µm fixed phase
Injection method	Splitless method high press mode 250 Kpa, 1.5 min
Injection time	1 min
Sample volumn	1 µl
Vaporization Tem.	230°C
Oven condition	50°C, 1 min; 15°C /min; 250°C, 3 min
Carries gas	He
Gas flow rate	Constant flow 5.19 cm/s
Mass spectrometry (MS) condition	
Ions face Tem.	230°C
Ions Tem.	200°C
Ionization method	SIM
Monitor ions	111, 112, 125
Event time	0.2 s

Table 2 Primers and probe used in this study

Primers and probe	Sequences (5'-3')	References
968F-GC	CGCCCGGGGCGCGCCCGGGCGGGGCGGGG GCACGGGGGG-AACGCGAAGAACCTTAC	21), 23)
1401R	CGGTGTGTACAAGGCCCGGGAACG	
BACT1369F	CGGTGAATACGTTTCYCGG	
PROK1492R	GGWTACCTTGTTACGACTT	
Probe TM1389BACT2	FAM-CTTGTACACACCGCCCGTC-TAMRA	

MD-1²²). Results of the 16S rDNA were linear between 2.67×10^0 and 2.67×10^6 copies μL^{-1} with R^2 values of 0.99. qPCR were performed in triplicate and each 20 μL of reaction mixture containing primer 0.3 μM and probe 0.2 μM at final concentration, 10 μL of THUNDERBIRD Probe qPCR Mix, 0.04 μL of 50 $\times$ ROX reference dye (Tyoko Co. Ltd., Osaka, Japan) and 1.0 μL of each DNA standard or sample template. Thermal profile consisted of an initial denaturation at 95°C for 1 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 56°C for 45 s. The increase in fluorescent signal was measured at each annealing step. The real-time PCR was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, USA).

ATP analysis ATP standard curve was prepared from a serial dilution of standard ATP solution. Mixing 100 μL BacTiter-Glo™ Reagent (Promega Corp., Madison, WI) and 100 μL subsamples or standard in each hole of an opaque-walled multiwell plate and incubated for 1 min to insure no lysis required to release ATP. Then luminescence was measured by Fluoroskan Ascent type 374 multiwell plate reader (LabSystems, Helsinki, Finland).

PCR-DGGE analysis 16S rDNA fragment of bacteria was amplified by PCR using DGGE universal primer set 968F- GC/1401R²³ (Table 2). The reaction mixture was 20 μL containing as, 1 μL of template DNA, 0.5 μL of each primer (20 μM), 2 μL of 10 $\times$ PCR buffer for Ex Taq (20 mM Mg^{2+} plus), 1.6 μL of dNTP mixture, and 0.25 μL of Ex Taq DNA polymerase (TAKARA BIO INC. Japan). PCR was performed with a Veriti 200 PCR Thermal Cycler (Applied Biosystems, Foster city, USA). The thermal profile for amplification was: 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and final 7 min at 72°C.

Products of PCR reaction were analyzed by subsequent DGGE based on the protocol of Muyzer et al.²⁴. PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 $\times$ TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH=7.4). To separate the amplified PCR fragments, the polyacrylamide gels were made with denaturing gradients ranging

from 45% to 55%. On each gel, a DGGE marker (DGGE Marker I, NIPPON GENE, Japan) was loaded, which was required for processing and comparing the different gels. The electrophoresis was run for 12.5 h at 60°C at a constant voltage of 80 V, which was performed by D-Code system (Bio-Rad, Hercules, CA). Following electrophoresis, the gel was stained for 1.0 h in Nucleic Acid Gel Stain (Gel-Red, Biotium, Hayward, CA) solution in 1 $\times$ TAE.

The obtained DGGE fingerprint were subsequently normalized and analyzed with the Quantity One software version 3.1 (Toyobo, Tokyo, Japan). During this processing, the different lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization, and the relative quantity was calculated, and the data were analyzed for PCA. A covariance data was extracted with pairwise deletion and varimax factor rotation. Data reduction provided a two-factorial ordering of the variance of DGGE profiles, which was plotted as a schematic diagram.

Statistics analysis A one-way ANOV and principal components in the statistical package for Social Science v17.0 software (SPSS Inc IL, USA) was applied to determine if there were significant differences between carbon sources and analyze principal components of DGGE fingerprint. Statistical significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Geosmin degradation under various carbon source conditions Figure 1 shows the time course of geosmin concentration change with natural biofilm in the presence of several organic carbon sources. Reduction of geosmin was observed in control which contributed to abiotic impact, like volatilization and adsorption. Reduction of geosmin was observed in control associating with abiotic impact, like volatilization and adsorption (Fig. 1). For only geosmin (biofilm with geosmin), there was no obvious lag phase and geosmin was gradually degraded within 4 days. The final geosmin removal was 99% (including abiotic loss) in only geosmin condition, which was agreed with the study

Fig. 1 Decrease in geosmin concentration during incubation of natural biofilm with several organic carbon sources.

◇: Control; sterilized biofilm with geosmin, ○: Only geosmin; biofilm with geosmin, △: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC. Bars represent the standard errors of the means for triplicates.

of Xue et al.²⁵⁾ that final geosmin removal was more than 90% in most months of the year. This result indicated indigenous bacteria in the biofilm still had ability to degrade geosmin; even geosmin concentration in influent water at water treatment plant was negligible. Geosmin biodegradation rate constant (K) was 0.55 d^{-1} ($R^2=0.95$), which was a pseudo-first-order reaction. As Andreozzi et al.²⁶⁾ reported that when organic compounds presented as secondary substrate, the degradation mechanism was pseudo-first-reaction. Hoefel et al.²⁷⁾ previously reported the rate constant of geosmin degradation by single bacterium Geo48. In their study, the rate constant was from 0.1 d^{-1} to 0.58 d^{-1} for initial geosmin concentration at 500 ng l^{-1} .

Glucose and NaAc had same geosmin removal efficiency (99%) as only geosmin condition (Fig. 1). There was no lag phase in the case of adding glucose and NaAc. Once experiment started, geosmin was rapidly degraded to below 10 ng l^{-1} within 2 days. Figure 1 showed the same decrease pattern under glucose and NaAc condition. This result indicated that existence of glucose or NaAc stimulated geosmin biodegradation. Geosmin biodegradation rate constant (K) was 1.35 d^{-1} ($R^2=0.90$) and 1.19 d^{-1} ($R^2=0.92$) for glucose and NaAc, respectively. These

rates were obviously higher than the rate of only geosmin and the results of other studies^{17),27)}. On the other hand, these rates were consistent with study of Saito et al.¹⁸⁾ who documented that geosmin biodegradation was accelerated through adding ethanol, which was base on the concept of co-metabolism. Co-metabolism is defined as transformation of a non-growth substrate by growing cells in the present of a growth substrate, by resting cells in the absence of a growth substrate, or by resting cells in the presence of an energy substrate²⁸⁾. We suspected that co-metabolic enzymes and cofactors were induced by glucose and NaAc.

HAC showed in evident geosmin biodegradation inhibition, compared with only geosmin condition (Fig. 1). Final geosmin removal was just 59%, which indicated that geosmin biodegradation was repressed by HAC. However, geosmin biodegradation rate constant was 0.52 d^{-1} ($R^2=0.99$) in the first day, which suggested geosmin was degraded in first day, but immediately repressed. This result might be an evidence for hypothesis that geosmin was degraded by a pathway similar to that of cyclohexanol. Trudgill²⁹⁾ documented that strains of *Acinetobacter* and *Nocardia* were capable of degrading cyclohexanol via monooxygenase enzymes, similar to the biological Baeyer-Villiger reaction³⁰⁾. Cyclohexanol was initially oxidized to an alicyclic ketone. The insertion of a ring oxygen atom followed via a monooxygenase enzyme. The resultant lactone was unstable, causing the lactone to be hydrolyzed into a diacid. HAC might hold-up this hydrolysis reaction, HAC or accumulated lactone caused some damage to geosmin degradation bacteria in this study.

Change of bacterial number and activity during geosmin biodegradation 16S rDNA copy number of only geosmin condition increased from 0 to 2nd day, then decreased gradually during incubation period (Fig. 2) Change of ATP concentration showed similar trend under only geosmin condition (Fig. 3), and the correlation coefficient (R^2) of geosmin concentration and ATP concentration was 0.93 ($p<0.05$). On the other hand, correlation coefficient (R^2) of geosmin concentration and 16S rDNA copies number was 0.83 ($p<0.01$).

These results indicated that geosmin in only geosmin condition was not degraded as growth substrate and energy substrate.

16S rDNA copy number of glucose and NaAc condition was not changed dramatically during the experiment (Fig. 2). On the other hand, glucose and NaAc had the same trend of ATP concentration change (Fig. 3), which stimulated bacterial activities within 2 days and the highest level were 201 pmol mL⁻¹ and 134 pmol mL⁻¹ of glucose and NaAc, respectively. It could be concluded that this two carbon sources stimulated bacterial activity, and rapid geosmin biodegradation was occurred, and geosmin concentrations decreased less than 10 ng L⁻¹ at the 2nd day. When bacterial activity decreased from the 2nd day, geosmin degradation also decreased. These results indicated glucose or NaAc existence just stimulated bacterial activities, but did not stimulate bacterial growth, so glucose and NaAc were utilized by bacteria as energy substrates. An energy substrate is defined as an electron donor that provides reducing power and energy, but does not by itself support growth, while a growth substrate is defined as an electron donor that provides reducing power and energy for cell growth and maintenance²⁸⁾. Obviously, the biodegradation of geosmin in this experiment was supposed to be induced by enzymes and cofactors produced by bacterial utilization of glucose or NaAc.

It is showed that 16S rDNA copy number of HAC condition slightly fluctuated in the first two days, and then increased until end of experiment (Fig. 2). Geosmin concentration kept stable during 16S rDNA copy number increased. This interesting phenomenon indicated that the indigenous bacteria possess ability of degrading geosmin, but after one day, some byproducts or factors produced by HAC utilization might inhibit geosmin biodegradation. This factor could be acid, because ATP could not be detectable from the 2nd day in HAC condition. Compared with only geosmin condition, HAC stimulated bacterial growth (Fig. 2), and this result further proved hypothesis that geosmin biodegradation pathway was same as cyclohexanol, which was degraded to hydrolysable diacid.

Fig. 2 Change of 16S rDNA copy number during incubation of natural biofilm with several organic carbon sources.

○: Only geosmin; biofilm with geosmin, △: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC.

Fig. 3 Change of ATP concentration during incubation of natural biofilm with several organic carbon sources.

○: Only geosmin; biofilm with geosmin, △: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc. The ATP concentration of HAC was undetectable because of low pH from day 2. Bars represent the standard errors of the means for triplicates.

DGGE fingerprint The PCA analysis using DGGE fingerprints indicated that significant difference of microbial community structures were observed between only geosmin and additional carbon sources condition (Fig. 4). The PCA plots of only geosmin were mainly located at right area, while the plots of glucose, NaAc and HAC were located at left area in the figure. It is

Fig. 4 Principal component analysis of the relative quantity generated by lane profiling for DGGE based upon 16S rDNA.

PCI 1 represents the first principal component explaining 16% characteristic of samples. PCI 2 represents the second principal component explaining 14% characteristic of samples. PCI 1 and PCI 2 are uncorrelated. The number of 0, 1, 2, 4, 6, and 8 is incubation day. ○: Only geosmin; biofilm with geosmin, △: Geosmin + glucose; biofilm with geosmin and glucose, ×: Geosmin + NaAc; biofilm with geosmin and NaAc, □: Geosmin + HAC; biofilm with geosmin and HAC.

indicated that additional carbon sources had significant effects on changing bacterial community structures during geosmin biodegradation.

Various environmental factors regulated bacterial community structures such as pH, carbon source species and concentration of bacterial metabolites³¹⁾. The complexity level of bacterial diversities was as follow; only geosmin > glucose and NaAc > HAC (data not shown). Consequently, it can be concluded that additional carbon sources such as glucose, NaAc and HAC decreased bacterial diversity in biofilm.

As Decho³²⁾ documented that cells within a biofilm can more easily adapt to changing environmental conditions. Figure 4 shows bacterial community structures changed dramatically from day 0 to day 2 at the all incubation condition. These were comparable with the study of Xue et al.²⁵⁾ that documented bacterial community structures dramatically changed within first 2 days.

CONCLUSIONS

The following conclusions can be drawn from this study results:

- (1) Geosmin degradation by natural biofilm was stimulated by adding glucose or NaAc at μg level concentration, while HAC repressed geosmin biodegradation.
- (2) Geosmin biodegradation seems to be induced by enzymes and cofactors produced by bacterial utilization of glucose or NaAc as energy source. Inhibition of HAC on geosmin biodegradation might be an evidence of hypothesis that geosmin biodegradation pathway is same as cyclohexanol.
- (3) This study might be showed that co-metabolic degradation of geosmin occurred in nature, because the biofilm in water treatment plants could degrade geosmin under presence of NOM.

ACKNOWLEDGEMENTS

The authors gratefully thank Shintaro Yamaguchi, Mariko Hirokawa, Xin Wang and Yu Gao for their technology and knowledge assistance. This work was partially supported by a Grant-in-Aid for Scientific Research (B) (25281042) from the Japan Society for the Promotion of Science.

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- (Submitted 2013. 5. 13)
(Accepted 2013. 6. 7)