chromatography (HPLC) equipped with hydrophilic interaction chromatography (HILIC).

2.1.1 Quantitative determination of 1,4-dioxane

Amounts of 1,4-dioxane were determined by spectrophotometric detection at 200 nm using an HPLC system. Acetonitrile-0.1% phosphate solvent system was applied as the eluent at 1 mL/min. Following injection of 40 µL of sample solution, 1,4-dioxane was separated by HPLC equipped with the HILIC column (TSKgel Amide-80 HR 4.6 i.d. 9 250 mm, 5µm particle size; Tosoh Corporation, Tokyo, Japan) at 25°C. BSM broth containing various amounts of 1,4-dioxane (5, 20, 50, 100, 250, 500, and 1000 mg/L) was used for calibration [6].

2.2 How the environmental polluting chemical compounds such as 1,4-dioxane are degraded by marine systems?

Environmental pollution by chemical compounds such as 1,4-dioxane has begun influencing marine systems. Hence, it is important to develop methods to measure biodegradation of chemical compounds such as 1,4-dioxane in marine systems and clarify the presence of 1,4-dioxane degrading microbes in such systems so that they can be applied for bioremediation.

2.2.1 Selection of microbes degrading 1,4-dioxane from seawater using a seawater-charcoal perfusion apparatus

To select microbes degrading 1,4-dioxane, seawater samples were obtained from four coastal areas of the Okinawa islands. and placed in the perfusion apparatus (Fujiwara Scientific Co., Ltd. Tokyo, Japan). Autoclaved Charcoal-A130 (Toyo Denka Kogyo, Kochi, Japan) as a microhabitat was dipped into each seawater sample for 12 h. The dipped charcoal (10 g) was set in a perfusion apparatus. The enrichment culture was carried out under dark conditions at 25°C by circulating 250 ml of BSM containing 1000 mg/L 1,4-dioxane [6]. The medium was circulated with air lift using an air pump through charcoal layer in the perfusion apparatus. The perfusion rate of the medium was adequately controlled by the air pump, and smooth leaching was maintained. The 1,4-dioxane concentration in culture fluids was monitored by HPLC during incubation. When the 1,4-dioxane concentration in culture fluids was decreased by almost 100 mg/L, the medium was replaced. After 1 month of incubation, the charcoal was detached from the equipped perfusion apparatus and the enriched charcoal (1 g) was crushed and suspended in 50 mM phosphate buffer (pH 7.0); the same buffer was added to the suspension to formulate a 10^{-4}-fold dilution. The diluted suspension was then inoculated on a BSM agar plate containing 1000 mg of 1,4-dioxane liter^{-1}. After 10 days incubation at 25°C, the colonies formed on the agar plate were isolated. Each colony detected on the culture plate was then inoculated and aerobically pre-incubated at 25°C by shaking with 5 mL BSM broth medium [6].

2.2.2 Degradation of 1,4-dioxane

The degradation test was performed using 20-mL aliquots of BSM broth medium (final 1,4-dioxane concentration: 1000 mg/L) after inoculation with 5 mL of pre-incubation broth as described above. Cultures were incubated statically at 25°C in the absence of light for 5 days. As a control, cultures were autoclaved at 121°C for 20 min following pre-incubation. At each sampling period, 0.1 mL of the culture broth was homogenized with 0.9 mL of acetonitrile and after centrifuged at 1500 × g for 10 min. The concentration of 1,4-dioxane in the supernatant was determined by HPLC system described above.

2.2.3 Identification of microbes degrading 1,4-dioxane

The DNA of microbes degrading 1,4-dioxane was sequenced by Techno-Suruga Laboratory Co., Ltd. (Shizuoka, Japan). The full 16S rDNA sequences were compared with reference sequences using BLAST similarity searches. The sequences were aligned using MAFFT. Evolutionary relationships were inferred using the neighbor-joining method. The phylogenetic tree was drawn using the NJplot WIN95 software.

2.2.4 PCR amplification test of soluble di-iron monooxygenase (SDIMO)

PCR amplification test using SDIMO primers determined whether the isolated strains would have the SDIMO α-subunit gene. It has been reported that some strains of 1,4-dioxane degrading bacteria have the SDIMO gene [7][8]. Primer sequences (5'→3') used for amplification test were (Forward) CAGTCNGAYGARKCSCGNCAYAT or CARATGYTNGAYGARGTNCGNCA and (Reverse) CGDATRTCRTCDATNGTCCA or CCANCCNGGRTAYTTRTTYTCRAACCA, referred from the previous reports [7][8]. DNA extraction from microbes and using these primers PCR amplification test were carried out according to the previous method [7].

3 Problem Solution

Use of the solvent gradient system with phosphate buffer containing 95% acetonitrile on an HPLC equipped with an HILIC column enabled certain amounts of 1,4-dioxane in the BSM broth medium to be determined. Therefore, this method was used for subsequent analyses.

As shown in Figure 1, the concentration of 1,4-dioxane in BSM broth medium repeatedly changed from almost 1000 mg/L to 100mg/L during incubation in the perfusion apparatus. Then, organisms were isolated from the charcoal attached to the perfusion apparatus used to culture water which showed the fastest 1,4-dioxane degradation speed. A total of almost 50 species of candidate microbes degrading 1,4-dioxane were selected as positive colonies on plates containing 1,4-dioxane and their degrading abilities were compared. Among these, RM-31 and KY-3 showed the strongest degrading ability and was therefore used for subsequent experiments.

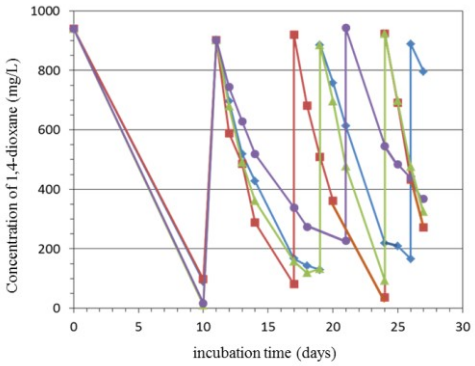

Fig. 1 Changes in concentration of 1,4-dioxane during culture in the perfusion apparatus with 4 seawaters from shores of Okinawa Islands.

Strain RM-31 decreased the level of 1,4-dioxane from the initial concentration of about 1000 mg/L to 700 mg/L within 2 days, after which it decreased slowly. The optimal initial pH and temperature were 6–8 and 25°C, respectively. The 1,4-dioxane degradation rate by strain RM-31and KY-3 in broth medium with 3% NaCl was almost 20% faster than that without NaCl (Fig.2).

Strain KY-3 decreased the level of 1.4-dioxne from the initial concentration of about 600 mg/L to 150 mg/L within 1 week, then OD 600nm increased slightly (Fig.3).

Strain RM-31 and KY-3 grew very slowly. The RM-31 cells formed hydrophobic colonies (flock) on the surface of the broth medium or on the glass of the tube containing the medium. Instead of the direct determination of cell growth, the protein production from microbes was measured. Using 5-mL culture broth with 800 mg/L of 1,4-dioxane concentration, the total protein extracted from the grown cell slightly increased from 0.034 to 0057 mg/tube during 3days-culture as residual 1,4-dioxane concentration decreased into about 400 mg/L. On the other hand, because KY-3 did not form flock KY-3 cell growth was determined by photo-absorbance at 600 nm.

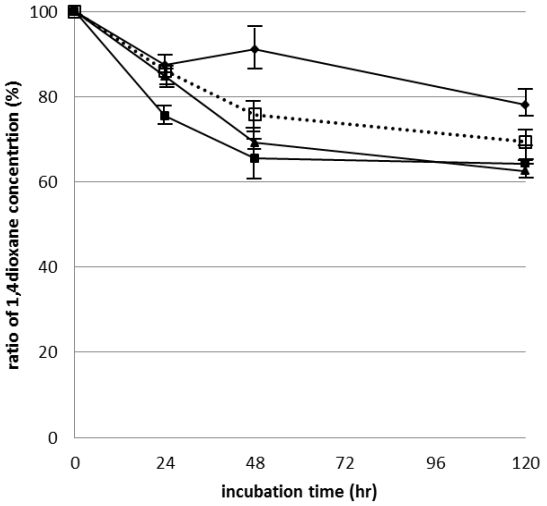

Fig. 2 Effects of incubation temperature and NaCl concentration on degradation of 1,4-dioxane (1000 mg/L) by *Pseudonocardia* sp. RM-31. The residual ratio (%) of 1,4-dioxane on broth medium with 3% NaCl at 20°C (closed triangles), 25°C (closed squares), 30°C (closed diamonds), and without NaCl at 25°C (open squares).

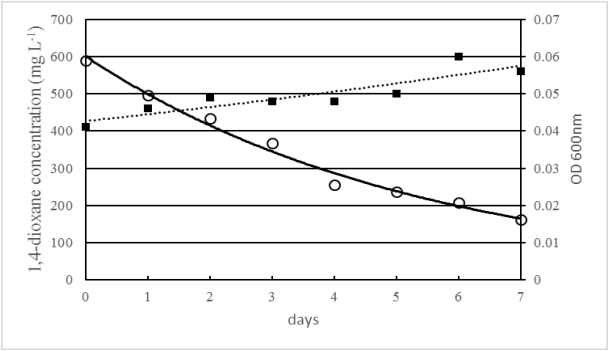

Fig. 3 Changes in the 1,4-dioxane concentration and cell growth during incubation of 5-ml BSM broth inoculated strain KY-3. The residual concentration of 1,4-dioxane (open circle), cell growth (closed square).

As culture incubation times increasing, the both residual 1,4-dioxane concentration was decreasing and the total protein and cell growth were increasing. It was suggested that RM-31 and KY-3 strain could

make degradation of 1,4-dioxane, following to the protein production by cell growth.
Similarity analysis using BLAST suggested that strain RM-31 was *Pseudonocardia carboxydivorans*. KY-3 was also identified as *Rhodococcus erythropolis* (homology 100%) by BLAST analysis. Figure 4 shows a phylogenetic tree comparing the sequence of strain RM-31 with similar sequences.

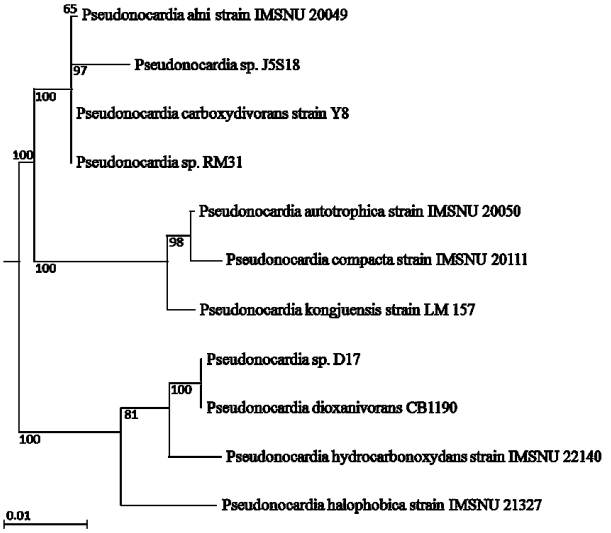

Fig. 4 Phylogenetic tree based on analysis of full 16S rRNA sequences of *Pseudonocardia sp.* RM-31 and their closest related sequences within *Pseudonocardia* sp.

PCR amplification test using SDIMO primers indicated that isolated strains had the SDIMO α-subunit gene.

4 Conclusion

To determine the concentration of 1,4-dioxane during the biodegradation process, a new method using HPLC equipped with an HILIC (hydrophilic interaction chromatography) column was developed. The developed method enabled easy and rapid determination of 1,4-dioxane and selection of 1,4-dioxane-degrading microbes. Two microbes that possessed 1,4-dioxane-degrading abilities were isolated from surface seawater using a seawater-charcoal perfusion method, among which strain RM-31 and KY-3 exhibited the strongest 1,4-dioxane-degrading ability. Sequencing of the 16S rDNA of strains selected and subsequent similarity analysis using BLAST indicated that strain RM-31 and KY-3 was identified as *Pseudonocardia carboxydivorans* and *Rhodococcus erythropolis* respectively. They are the first bacteria from seawater shown to have a strong ability to degrade 1,4-dioxane in seawater. The average degradation rate by strain RM-31 was about 6.25 mg L^{-1}/h and KY-3 was 3.86 mg L^{-1}/h during 2days. They showed the optimal initial pH and temperature being 6–8 and 25°C, respectively. The biodegradation rate of 1,4-dioxane by strain RM-31 and KY-3 at 25°C in broth medium with 3% NaCl was almost 20% faster than that without NaCl. PCR amplification test using SDIMO primers indicated that isolated strains had the SDIMO α-subunit gene. These findings confirm the existence of 1,4-dioxane degraders in seawater and indicate that they have the potential for application to bioremediate this compound.

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