Taxonomic Characteristics and Anticancer Activity of Actinomycetes Isolated from the Marine Environment of Izu Akazawa

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Abstract

We isolated 100 actinomycete strains each from deep-sea water (DSW) at 800 meters depth, surface seawater (SSW) near the DSW, and intertidal zone sediments (IS) in Sagami Bay. We performed morphological identification of these strains and investigated their growth temperature range, NaCl tolerance, pressure tolerance, hexavalent chromium tolerance, antimicrobial activity, and anticancer activity.

The results showed that more than 90% of the isolated actinomycetes from SSW and IS belonged to the genus *Streptomyces*. In contrast, nearly 30% of the actinomycetes isolated from DSW were from the genus *Micromonospora*. The strains exhibited no significant differences in growth at temperatures between 15°C and 37°C, with over 80% of strains from all sources being capable of growth within this temperature range. However, at the low temperature of 4°C, about 20% of strains from DSW and SSW were capable of growth, while only 2% of strains from IS were capable of growth. NaCl tolerance tests revealed that the strains from IS, SSW, and DSW exhibited increasing levels of tolerance, respectively. Pressure tolerance assessments showed that many strains from DSW demonstrated good growth under high-pressure conditions. Regarding hexavalent chromium tolerance, strains from IS exhibited the highest resistance, followed by strains from SSW and then DSW. Antimicrobial activity was most prevalent among strains from SSW, followed by those from DSW and then IS. When evaluating anticancer activity using human-derived colorectal cancer cells (A549 cells) and human-derived lung cancer cells (HT-29 cells), 11 strains (11%) and 12 strains (12%) from DSW, 17 strains (17%) and 17 strains (17%) from SSW, and 10 strains (10%) and 10 strains (10%) from IS showed anticancer activity. SSW strains had the highest positive rate for anticancer activity. Given the significant differences in the characteristics of actinomycetes isolated from different sources, we plan to focus on actinomycetes derived from DSW in future studies to elucidate their metabolic products and further explore their potential.

Key words : actinomycete, deep seawater, *Streptomyces, Micromonospora*

1. Introduction

Actinomycetes are Gram-positive bacteria known for their complex morphological differentiation (Laskin et al., 1973). They are renowned for producing a wide variety of bioactive compounds; about two-thirds of the approximately 10,000 low-molecular-weight bioactive substances discovered so far are produced by actinomycetes (Hu et al. 2015). Specifically, as of 2005, 801 out of 989 known anticancer, substances were derived from actinomycetes, accounting for more than 80% of the total (Bérdy et al., 2005). Besides the diversity of their products, actinomycetes are distinguished by their complex morphological differentiation and have been treated as valuable microorganisms. Although actinomycetes are originally thought to be terrestrial microbes, they are known to adapt to various environments

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(Ngamcharungchit et al., 2023). Many actinomycetes have been isolated and studied for their properties and products (Mikami et al., 2010, 6Stackebrandt et al., 1997). However, with the discovery of numerous new bioactive substances from soil microorganisms, the rate of discovery of new actinomycete-t derived substances has been decreasing in recent years (Busti et al., 2006). Consequently, research on actinomycetes from unique environments, such as marine environments with high salinity and low nutrients (Chen et al., 2006), animal hosts (Takahashi et al., 1996), and plant symbioses (Igarashi et al., 2004), has been increasing.

Marine environments are known to harbor a wide variety of microorganisms, including marine bacteria, which have been reported to produce various bioactive substances such as anticancer agents (Maskey et al., 2003), antibiotics (Hotta et al., 1980), and cytotoxic compounds (Kanoh et al., 2005). Additionally, it has been reported that microbial community composition differs among marine environments such as marine sediment, surface seawater, and deep-sea water (Terahara et al., 2016), indicating potential for discovering new bioactive substance-producing microorganisms from various marine environments (Panchanathan et al., 2014). Furthermore, actinomycetes grown in unique environments may produce bioactive substances not previously observed due to changes in their metabolic pathways (Okazaki et al., 1975). However, the history of isolating actinomycetes from marine environments is relatively short compared to terrestrial environments, and the ecology of marine-derived actinomycetes remains largely unexplored due to the challenges of sampling.

In this study, we focused on Sagami Bay in the Japanese coastal waters and isolated actinomycetes from three types of marine environments: deep-sea water at a depth of 800 meters (referred to as DSW), surface seawater (referred to as SSW), and intertidal soil (referred to as IS). We investigated the growth temperature range, NaCl tolerance, pressure tolerance, and hexavalent chromium tolerance of the isolated strains to elucidate the differences between DSW-derived strains and others. Additionally, we assessed the production of antimicrobial and anticancer substances.

2. Materials and Methods

2-1. Collection of Marine Samples

In this study, we received bag filters (referred to as BF) used for removing suspended solids from deep-sea water (DSW) at 800 m depth from the Izu Akazawa Deep-Sea Water Intake Facility (DHC Co., Ltd.) in Ito City, Shizuoka Prefecture. Although DSW is typically very clean and contains few microorganisms, filtering large volumes of DSW using these filters allows for efficient collection of microorganisms. For comparison, we also collected surface seawater (SSW) from the vicinity of the DSW intake facility and intertidal zone sediments (IS) from a nearby location, resulting in a total of three types of marine samples. The BF and SSW samples were stored at 4°C, while the IS samples were stored at -80°C until the actinomycete isolation procedures. SSW was collected in 3 liters, filtered through a membrane filter (Supor-200 Membrane Filter 3.0μm 47mm PALL Life Science), and then further filtered through a membrane filter with a smaller pore size (Supor-200 Membrane Filter 0.2μm 47mm PALL Life Science), with each filter used as a source for actinomycete isolation.

2-2. Isolation of Actinomycetes from Marine Samples

Given the expected low number of actinomycetes relative to other microorganisms in marine samples, it was deemed necessary to eliminate the majority of contaminating bacteria before isolating actinomycetes. Each sample (1g for IS, 3cmx3cm for DSW, and one filter for SSW) was suspended in 20 ml of sterilized DSW, followed by heat treatment at 55°C for 30 minutes to eliminate contaminating marine bacteria (Hoskisson et al., 2000). After treatment, 100 μl of the suspension was spread onto four types of media prepared with distilled water (DW) or DSW: ISP-4 and HV media. To selectively isolate actinomycetes, nalidixic acid (20 μg/ml) and cycloheximide (50 μg/ml) were added as anti-Gram-negative bacterial agents and antifungal agents, respectively. After incubation, the number of microbial colonies on each medium was counted to determine the selective isolation rate of actinomycetes. The isolation rate was calculated using the following formula:

Selective Isolation Rate (%) = (Number of actinomycete colonies / Total number of microbial colonies) × 100

2-3. Morphological Identification of Isolated Strains

Actinomycetes were isolated from BF using ISP-4 (DW) and HV (DW) media. After confirming sufficient growth on ISP-2 (DW) medium, the strains were preserved on the same medium. For comparison, 100 actinomycete strains each

were also isolated from SSW and IS, totaling 300 strains. The isolates were classified into the genera *Streptomyces* and *Micromonospora* based on morphological characteristics. Specifically, large colonies starting white to gray and becoming covered with powdery aerial mycelium over time were classified as **Streptomyces*, while smaller, firmer colonies forming yellow or orange spores that turn black or brown over time were classified as *Micromonospora*.

2-4. Measurement of Growth Temperature Range

Each of the 100 strains isolated from the three sources was inoculated onto ISP-2 medium (DW) and incubated at temperatures of 4°C, 15°C, 20°C, 27°C, 30°C, 37°C, and 50°C. Growth was assessed to determine the temperature range for growth. Cultures were incubated for one month at 4°C and for one week at other temperatures.

2-5. NaCl Tolerance Test

ISP-2 medium (DW) with final NaCl concentrations of 0%, 1.5%, 3%, 6%, 9%, 12%, and 15% was prepared. After inoculating the test strains onto these media, they were incubated at 27°C for one week, and growth was visually assessed.

2-6. Pressure Tolerance Test

Each isolated strain was inoculated into 10 ml of ISP-2 liquid medium (DW) and divided equally into two sterile sampling bags (3”×5”, Fisher Scientific). One bag was placed in a pressure cylinder (PV-100 High-Pressure Container, Shinco Corporation) and pressurized to 8 MPa, then incubated at 27°C for three days. The other bag was incubated under normal pressure (0.1 MPa) for comparison. After incubation, growth was measured based on microbial DNA content. DNA was extracted from the microbial cells using the SDS method, and absorbance at $\lambda = 260$ nm was measured to evaluate growth (Ozcengiz et al., 2000; Tamburini et al., 2004).

2-7. Hexavalent Chromium Tolerance Test

ISP-4 medium (DW) containing K2CrO4 at final concentrations of 50 mg/l and 100 mg/l was prepared. Test strains were inoculated and incubated at 27°C for two weeks. Growth was assessed visually.

2-8. Antimicrobial Activity Test

Antimicrobial activity of the test actinomycetes was assessed using the paper disk method. Cultures of test strains were prepared in 24-well plastic plates (Costar) with 1.5 ml of ISP-2 liquid medium (DW) per well and incubated at 27°C with shaking at 380 rpm for one week. After centrifugation (16,000 × g, 10 minutes), the supernatant was used for antimicrobial activity testing. Fifty µl of the supernatant was applied to paper disks (ADVANTEC, 6 mm diameter) placed on the test microorganism media and incubated at 27°C for one day. Gram-positive bacterium *Bacillus subtilis* PCI219, Gram-negative bacterium *Escherichia coli* K-12, and fungus *Candida albicans* 3147 were used as test microorganisms. After autoclaving, media were cooled to below 60°C, and test microorganism suspensions were added to achieve a final concentration of 1%. Antimicrobial activity was assessed based on the presence of inhibition zones around the paper disks.

2-9. Anticancer Activity

Anticancer activity was evaluated using human-derived colorectal cancer cells (A549 cells) and human-derived lung cancer cells (HT-29 cells) with a total of 289 actinomycete strains: 98 from DSW, 99 from SSW, and 92 from IS. The MTT reduction method described in section 2.10 was used to assess cell inhibition effects. The culture supernatants of the test strains (see section 2.11) were added to plates with cells at a final concentration of 5% and cultured. Strains that reduced cell viability to below 50% of the control were considered positive for anticancer activity. The evaluation was performed with $n = 1$, and positive controls included 0.4 µl/ml SN-38 (Cayman Chemical) and 0.02 µl/ml docetaxel (Sanofi Co., Ltd.).

2-10. MTT Reduction Method for Cell Inhibition

Eagle's MEM (Nippon Shinyaku) containing 10% fetal bovine serum (FBS, Biological Industries) and 200 mM glutamine was used as the base medium. Culture supernatants of each actinomycete strain (see section 2.11) were added to achieve a final concentration of 10% in the medium. Cells were seeded at $1.5 - 2.0 \times 10^4$ cells/well in a 96-well microplate and pre-cultured for one day at 37°C with 5% CO_2. After pre-culturing, the base medium with a final

concentration of 10% culture supernatant was added, and cells were further cultured for two days at 37°C with 5% CO_2. Negative controls (NC) used base medium without culture supernatant, adjusted to a final concentration of 5%. After the evaluation culture, cell activity was measured using the MTT reduction method. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to a final concentration of 5% and incubated at 37°C for 2 h. After removing the medium, 100 µl of 0.04N HCl in 2-propanol was added to extract formazan, followed by absorbance measurement at λ = 570 nm - 655 nm to evaluate cell inhibition effects (Moman et al., 1983). Actinomycete strains were considered positive if they showed less than 50% absorbance compared to the NC. Data were expressed as mean ± standard deviation.

Cell inhibition effect = (Absorbance of test sample at λ = 570 nm - 655 nm) / (Absorbance of NC at λ = 570 nm - 655 nm) × 100

3. Results and Discussion

3.1. Examination of Actinomycetes Isolation Media

As shown in Table 1, few colonies that appeared to be actinomycetes emerged on media using DSW (Deep Seawater). This suggests that actinomycetes are originally terrestrial【Hodges et al., 2012】, and few actinomycetes require DSW for growth. The most effective medium for isolating actinomycetes from DSW was ISP-4 medium (DW). However, colonies presumed to be actinomycetes were also isolated from HV medium (DW), with some colony morphologies not observed on ISP-4 medium. The effectiveness of HV medium (DW) is supported by reports that it yields a greater number of non-*Streptomyces* species colonies【Ruddick, and Williams, 1972】. Based on these results, we decided to use both HV medium (DW) and ISP-4 medium (DW) for actinomycetes isolation in this study to maximize isolation efficiency.

Table 1 Isolation rate of actinomycetes on different media

	ISP-4 agar		HV-agar	
	×10 ^{3} cfu/g			
	DW	DSW	DW	DSW
No. of actinomycete colonies	2.1	0	0.23	0
Total No. of colonies	5.2	0	1	0
Actinomycete isolation rate (%)	40.3	0	23	0

3.2. Actinomycetes Isolation Rate

As illustrated in Fig. 1, more than 90% of the isolated actinomycetes from SSW (Surface Seawater) and IS (Intertidal Sediment) belonged to the *Streptomyces* genus. However, while *Streptomyces* dominated the isolates from DSW, nearly 30% of the isolates were from the *Micromonospora* genus. *Micromonospora* is known to thrive under high-pressure conditions and has been frequently isolated from deep-sea environments【Mincer et al., 2002】. This is consistent with the actinomycetes isolation rate observed in DSW. Some microorganisms residing in deep-sea environments are known to proliferate more effectively under high pressure rather than atmospheric pressure【Jannasch and Taylor, 1984】, indicating the possibility of DSW harboring unique actinomycetes.

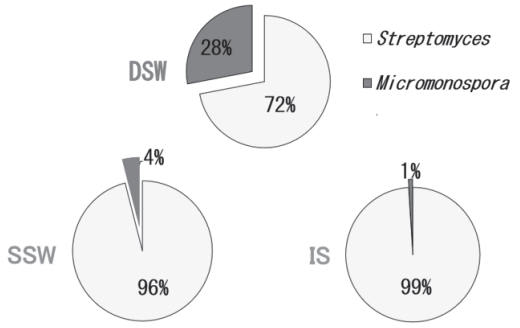

Fig. 1 Types of actinomycetes from different sources based on macroscopic identification

3.3. Growth Temperature Test of Isolates

For these isolates, no significant growth differences were observed across sources at temperatures between 15°C and 37°C, with over 80% of the actinomycetes able to grow at all temperatures (Fig. 2). However, at 4°C, around 20% of the actinomycetes from DSW and SSW were able to grow, whereas only 2% of those from IS showed growth. This indicates that actinomycetes from cold environments like DSW and SSW have higher cold tolerance compared to those from terrestrial sources. Conversely, under high-temperature conditions (50°C), nearly 40% of the actinomycetes from IS grew, while only 3% of those from DSW showed tolerance. This suggests that actinomycetes from DSW, which consistently resides at around 4°C, exhibit low resistance to high temperatures like 50°C.

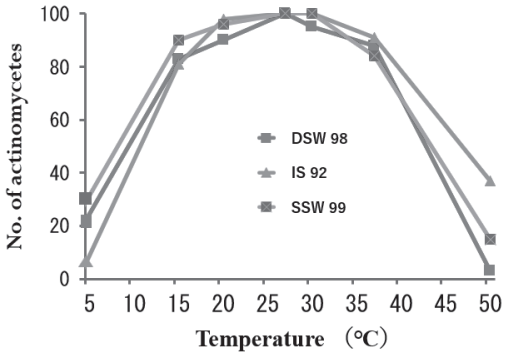

Fig. 2 Growth temperatures of actinomycetes from different sources

3.4. NaCl Tolerance Test of Isolates

As seen in Fig. 3, actinomycetes from IS, SSW, and DSW showed increasing tolerance to NaCl in that order. This contrasts with previous reports by Imada et al.【2010】. The NaCl tolerance of actinomycetes from IS exceeded by about 20% at NaCl concentrations of 3% and 6%. The IS used in this experiment was collected from intertidal sediment, where salinity fluctuations due to weather and tides are significant. This suggests that the high NaCl tolerance of the isolated actinomycetes was influenced by the elevated salinity in the sediment. Additionally, the low NaCl tolerance of actinomycetes from DSW is likely due to the high isolation rate of the *Micromonospora* genus. Generally, most actinomycetes isolated from soil belong to the *Streptomyces* genus【Hayakawa, 1990】, but nearly 30% of the isolates from DSW were *Micromonospora* (Fig. 1). Previous studies have reported that *Micromonospora* exhibits lower NaCl tolerance than *Streptomyces* 【Mincer et al. 2002】, which was confirmed in this study, as almost all *Micromonospora* isolates showed NaCl tolerance between 3% and 6%, lower than that of *Streptomyces.* Although actinomycetes were isolated from SSW and DSW, they only demonstrated NaCl tolerance below 3%. It is hypothesized that some actinomycetes may have existed in a spore state during their life cycle, allowing them to survive in environments without high NaCl resistance【Cross, 1981】.

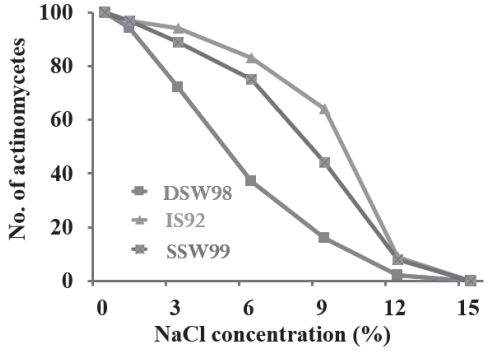

Fig. 3 NaCl tolerance of actinomycetes from different sources

3.5. Pressure Resistance Test of Isolates

A total of 289 actinomycete strains, 98 from DSW, 99 from SSW, and 92 from IS, were tested for pressure resistance. As shown in Fig. 4, the number of strains that grew better under high-pressure conditions than at atmospheric pressure was highest for DSW, followed by SSW and IS. DSW is constantly exposed to high water pressure, and the ability to

grow quickly under such conditions is thought to be advantageous for the survival of actinomycetes. Particularly, the stable environment of DSW suggests that actinomycetes from DSW have adapted over time to deep-sea conditions. Additionally, SSW circulates between the surface and the compensation depth of the ocean, so SSW-derived actinomycetes are exposed to moderate water pressure, which likely resulted in more strains that thrive under high-pressure conditions than those from IS.

Fig. 4 Pressure tolerance of actinomycetes from different sources

3.6. Hexavalent Chromium Resistance of Isolates

The hexavalent chromium resistance, used as an indicator of resistance to harmful substances, was highest for IS-derived actinomycetes at both 50 mg/L and 100 mg/L concentrations, followed by SSW and DSW (Fig. 5). Hexavalent chromium is only present in trace amounts in the natural environment, with human activities being the primary source of contamination. Hexavalent chromium released within environmental standards is easily reduced by organic matter in soils and rivers, and thus its concentration in marine environments is extremely low【Velma et al., 2009】. Therefore, actinomycetes with high hexavalent chromium resistance were scarce in DSW, an environment relatively free from contamination.

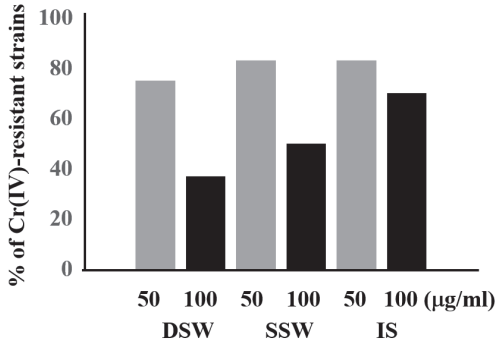

Fig. 5 Proportion of Cr(IV)-resistant strains for each source of isolation

3.7. Antimicrobial Activity of Isolates

As shown in Table 2, actinomycetes with antibacterial activity were most frequently isolated from SSW, followed by DSW and IS. None of the isolates exhibited activity against the gram-negative bacterium *Escherichia coli* K-12, while the highest number of actinomycetes showed activity against *Bacillus subtilis* PCI219 across all sources. There were no significant differences in the antibacterial activity results, but the actinomycetes from DSW displayed comparable production rates of antibacterial substances to those isolated from terrestrial soils, which have yielded many antibiotic-producing strains. This suggests that DSW holds great potential as a resource for discovering new antibiotic-producing bacteria.

Table 2 Antimicrobial activity of each isolate

Sample	No. of strains	*Bacillus subtilis* PCI219	*Escherichia coli* K-12	*Candida albicans* NBRC1594
DSW	100	7	0	1
SSW	100	9	0	1
IS	100	5	0	0

3.8. Anticancer Activity of Isolates

A total of 289 strains, 98 from DSW, 100 from SSW, and 93 from IS, were tested for anticancer activity against A549 and HT-29 cancer cell lines (Table 3). Anticancer-positive strains were identified in 38 strains (13%) and 39 strains (13%), respectively. When analyzed by source, 11 strains (11%) and 12 strains (12%) from DSW, 17 strains (17%) and 17 strains (17%) from SSW, and 10 strains (10%) and 10 strains (10%) from IS were found to be positive. The highest anticancer-positive rate was observed in SSW isolates. These rates were higher than those reported for actinomycetes isolated from terrestrial soils【Zheng et al., 2000】, indicating that the marine environment holds promise as a source for discovering new anticancer substances. Although DSW isolates showed fewer positive strains than SSW isolates, DSW isolates included a higher proportion of *Micromonospora* and exhibited better growth at low temperatures (4°C) and high-pressure conditions. Therefore, DSW isolates may possess unique metabolic systems and could serve as a source for discovering novel anticancer substances.

Table 3 Anticancer activity of each isolate

Source		No. of positive strains (%)	
		A549 cell	HT-29 cell
DSW	98 (100)	11 (11)	12 (12)
SSW	100 (100)	17 (17)	17 (17)
IS	93 (100)	10 (11)	10 (11)
Total	291 (100)	38 (39)	39 (40)

It is widely known that chemotherapy is one of the most frequently used treatments for cancer【Mohammad, and Lee, 2015】. However, limitations exist regarding the specificity and efficacy of anticancer agents, and serious side effects are a significant concern【Alam and Lee, 2016】. Additionally, drug-resistant cancer cells have emerged as a recent challenge【Wang et al., 2019】, highlighting the need for the discovery of new anticancer drugs with fewer side effects and high specificity against cancer cells. The marine environment has become a focus for such searches, leading to the discovery of a novel antitumor compound, salinosporamide, from the marine actinomycete *Salinispora tropicana*. Since then, the search for antitumor compounds from marine actinomycetes has intensified【Jagannathan et al., 2021】. However, no examples of such isolates have yet been reported from DSW. Therefore, further studies are needed to isolate and purify anticancer substances from DSW strains, analyze their structures, and evaluate their anticancer activities against various cancer cells.

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