Screening of Plant-Derived Actinomycetes with Antifungal Activity

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***Abstract*—**A screening procedure was implemented to identify actinomycetes that synthesize antifungal antibiotics. Plants indigenous to Hiroshima Prefecture were used instead of soil to diversify the source of actinomycete isolates, which has conventionally been utilized in previous studies. During the primary screening, putative actinomycetes isolated from plants were separated based on their biochemical traits, acquiring two hundred and eighty-two actinomycete strains. Subsequently, for the secondary screening process, five fungal strains (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus oryzae*, *Mucor hiemalis*, and *Rhizopus stolonifer*) were used to evaluate the antifungal activity. The findings revealed that, out of the two hundred and eighty-two strains, five actinomycetes demonstrated antifungal activity against all tested fungi. Lastly, a tertiary screening compared the antifungal effects of five actinomycetes demonstrating activity against all tested fungi. More specifically, a comparison was made regarding the potency of antifungal substances produced by the five strains acquired, using commercially available antifungal agent amphotericin B as an indicator. The results of tertiary screen confirmed that HIT18, among the five actinomycetes, possessed a broad antifungal spectrum.

***Keywords*—** Actinomycetes, Antifungal agent, Amphotericin B, grass blade.

I. Introduction

Numerous microorganisms have been discovered in nature, and although some are pathogenic, many are beneficial to humans, including those used for producing fermented foods and pharmaceuticals [1]. One such valuable microorganism is actinomycetes, which produce antibiotics as secondary metabolites. Many studies have been conducted to isolate actinomycetes for treating infectious diseases, and consequently, several efforts have been made to identify actinomycetes that produce novel antibiotics.

Many antibiotics isolated from actinomycetes exhibit antibacterial activity; however, only a limited number demonstrated efficacy against fungi. Recently, fungal diseases, such as aspergillosis and mucormycosis, have attracted attention, emphasizing the need for antibiotics to combat fungi and bacteria [2].

However, due to considerable similarity between fungi and mammalian cells, using exclusively effective antimicrobial agents against fungi without causing harm to the human body remains elusive [3]. Therefore, actinomycetes produce only a small number of antifungal agents that have been used commercially, including Nystatin, Natamycin, and Amphotericin B [4]. Amphotericin B, used for treating deep-seated fungal infections, is the most successful antifungal antibiotic. However, it has severe side effects [5]-[7]. Consequently, AmBisome, a liposomal antimicrobial agent, was developed to enhance therapeutic efficacy while minimizing the toxicity of amphotericin B. Nevertheless, it did not suppress adverse effects like fever, chills, and laxatives [8]. Therefore, identifying antibiotics with broad-spectrum antibacterial activity against fungi and minimal side effects constitutes a critical research priority. Actinomycetes have been predominantly isolated from soil samples [8],[9]. However, screening soil as an isolation source presents a challenging task for obtaining novel antibiotic-producing actinomycetes. Hence, this study aimed to obtain antifungal activity-containing actinomycetes by utilizing plants native to Hiroshima Prefecture as a source of isolation.

II. Materials And Methods

A. Primary screening

We used grass blades for isolating actinomycetes. The grass blades were collected from the Yawata River basin near the Hiroshima Institute of Technology. The isolation of actinomycetes from the collected plant samples was performed according to the experimental methodology of Hasegawa *et al*. [10]. After confirming the growth of actinomycetes, colonies were picked up using platinum ears and inoculated onto International *Streptomyces* Project-2 Medium (ISP-2) agar medium containing yeast extract (0.4%), malt extract (1%), D (+)-glucose (0.4%), and agar (20%) [11]. The isolation process was repeated until the microscopic observation confirmed the absence of contamination. The isolates were classified based on colony shape, pigment production, and mycelial morphology, as observed by microscopy. When determining which strains to store, we excluded strains with similar characteristics isolated from the same source.

B. Secondary screening

The antifungal activity of actinomycetes obtained from the primary screen was measured during the secondary screening. First, the actinomycetes were collected with a platinum ear and cultured in 2 mL of ISP2 medium at 30°C for 168 hours with shaking. Around 2 mL of the culture was transferred to 10 mL of ISP2 medium and incubated at 30°C for 168 hours with shaking. The culture medium was centrifuged at 4 °C, 1,000 rpm, for 15 minutes, and the culture supernatant was collected.

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The collected culture supernatant was assayed for antimicrobial activity by the paper disk method [12]. Five fungi were used to measure the antimicrobial activity, *Saccharomyces cerevisiae* HUT7107, *Schizosaccharomyces pombe* HUT7157, *Aspergillus oryzae* HUT2363, *Mucor hiemalis* HUT 1262, and *Rhizopus stolonifer* HUT2363. To prepare spore suspensions of *M. hiemalis*, *A. oryzae*, and *R. stolonifer*, these microorganisms were inoculated onto Potato Dextrose Agar "Nissui" containing (Nissui Pharmaceutical Co., Ltd.) as a slant medium and grown at 30°C until fully grown. Polyethylene (20) Sorbian monooleate (Fujifilm Wako Pure Chemicals Co., Ltd.) at 0.05% was added to the saline solution to obtain dilutions and added to the slant medium and suspended with platinum loops. The suspension was filtered through sterilized cotton wool and collected in a 15 mL centrifuge tube. The collected spore solution was diluted with saline to a 1.0×10^3 spores/mL spore count. To collect vegetative cells of *S. cerevisiae* and *Sch. pombe*, each yeast was cultured on 108 YM agar medium, containing yeast extract (0.3%), malt extract (0.3%), D (+)-glucose (1%), bacto peptone (0.5%), and agar (2%). The culture was incubated at 30°C until just before the stationary phase. Subsequently, 10 mL of saline solution was added to the cultured slant medium, and the bacteria were suspended with platinum loops. The suspended vegetative cell sap was observed under a microscope and diluted with saline to 5.0×10^3 cells/mL cell count.

The spore and vegetative cell suspensions were used to measure the antimicrobial activity, and the actinomycete culture medium's antimicrobial activity was evaluated using the paper disk method.

C. Tertiary screening

During the tertiary screening, actinomycetes selected in the secondary screen were evaluated, which were amphotericin B equivalents. Only strains that exhibited an antimicrobial spectrum equal to or greater than amphotericin B were evaluated were selected. The amphotericin B equivalents were measured as follows: Amphotericin B was diluted stepwise using dimethyl sulfoxide, and its antifungal activity against each indicator bacteria was measured similarly to the antifungal activity assay. A calibration curve was constructed using the size of the inhibition zone corresponding to the concentration of amphotericin B. The amphotericin B equivalents of the actinomycete cultures selected in the secondary screening were determined using the calibration curve, and actinomycete strains were selected.

III. Results And Discussions

A. Primary screening

Actinomycetes were isolated from grass blades from the Hiroshima Prefecture, yielding two hundred and eighty-two putative actinomycete strains. The strains showing indistinguishable visual and microscopic characteristics were excluded from further analysis. Among the two hundred and eighty-two strains, fifty-eight were gray, forty-eight yellow, twenty-four pink, thirty-one green, twenty brown, fifty-five orange, one black, and forty-five white.

B. Secondary screening

The two hundred and eighty-two strains obtained from the primary screen underwent the secondary screening. Forty-four actinomycetes exhibited antifungal activity against *S. cerevisiae* and thirty-nine strains against *Sch. pombe*. In addition, forty strains exhibited activity against *A. oryzae*, thirty-four strains against *M. hiemalis*, and twenty-two strains against *R. stolonifer*. In particular, HIT 10, HIT 18, HIT 55, HIT 121, and HIT 186 exhibited antifungal activity against all five tested strains, thus affirming their potential as antifungal agents. Furthermore, the cultural characteristics of these five strains are summarized in Table 1.

TABLE I: Cultivation Characteristics of Selected Five Strains

Characteristics	HT10	HIT18	HIT55	HIT121	HIT186
Color of the aerial mycelium	Light Gray	Yellow	White	Yellow	Yellow
Color of the substrate mycelium	Brown	Yellow	Orange	Yellow	Yellow
Pigmentation	Brown	Brown	-	Light brown	-

It was observed that yellow colonies with diffuse pigmentation were the predominant phenotype of the actinomycetes exhibiting antifungal activity. For example, the yellow colony with diffuse pigmentation; a photograph of the colony of HIT18 is shown in Fig. 1.

Fig. 1. The HIT18 colony on International *Actinomyces* Project-2 Medium agar plate

C. Tertiary screening

The antifungal potency per mL of broth of the five isolates that showed activity (HIT10, HIT18, HIT55, HIT121, and HIT186) was estimated as amphotericin B equivalents.

First, the results of evaluating the antifungal activity against *S. cerevisiae* of the five isolates in Fig. 2 are shown.

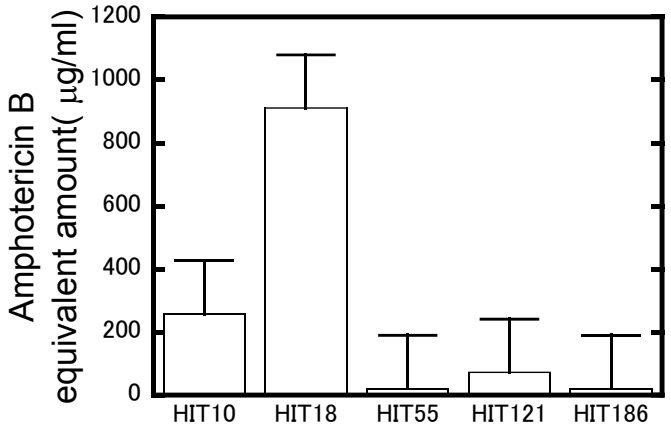

Fig.2. Amphotericin B equivalent of five actinomycete strains against *S. cerevisiae*

As shown in Fig. 2, HIT18 demonstrated the most potent antifungal activity against *S. cerevisiae* among the five actinomycetes, with an amphotericin B equivalent of 9.10×10^2 μg/mL against *S. cerevisiae*. HIT10 exhibited the second-strongest antifungal activity after HIT18, i.e., 2.56×10^2 μg/mL.

Fig. 3 shows the results of the evaluation of the antifungal activity of the five isolates against *Sch. pombe*.

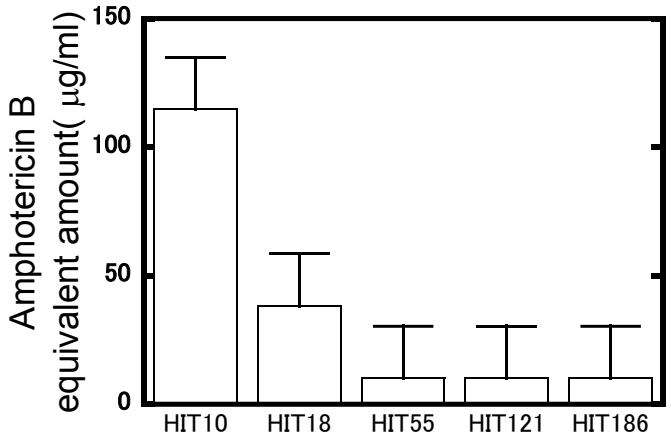

Fig.3. Amphotericin B equivalent of five actinomycete strains against *Sch. pombe*

As shown in Fig. 3, HIT10 exhibited the most potent antifungal activity against *Sch. pombe* among the five actinomycetes. HIT10 demonstrated an amphotericin B equivalent of 1.15×10^2 μg/mL against *Sch. pombe*.

HIT18 exhibited the second-highest antifungal activity after HIT10, i.e., 3.41×10 μg/mL.

Fig. 4 shows the evaluation results of the antifungal activity of the five isolates against *A. oryzae.*

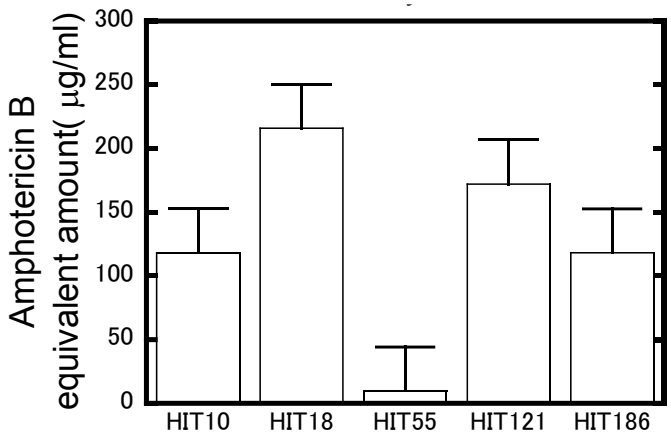

Fig.4. Amphotericin B equivalent of five actinomycete strains against *A. oryzae*

As shown in Fig. 4, among the five actinomycetes, HIT18 exhibited the most potent antifungal activity against *A. oryzae*. The antifungal activity of HIT18 was an amphotericin B equivalent of 1.09×10^4 μg/mL against *A. oryzae*. Other antifungal activities were 1.18×10^2 μg/mL for HIT10, 1.72×10^2 μg/mL for HIT121, and 1.18×10^2 μg/mL for HIT186. HIT55 had a weaker antifungal activity than the other candidate strains at 9.79 μg/mL.

Fig. 5 shows the results of the evaluation of the antifungal activity of the five isolates against *M. hiemalis.*

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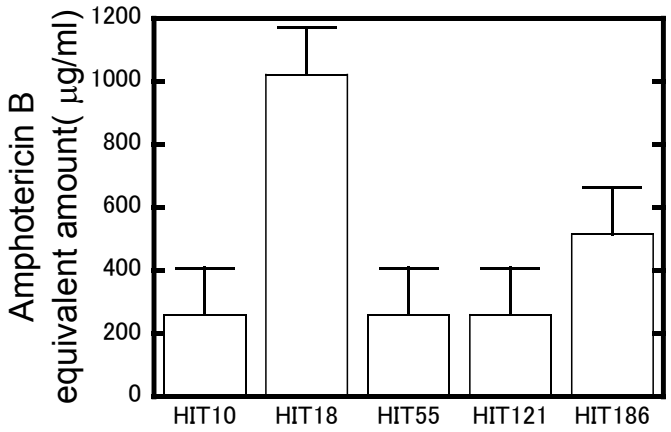

Fig.5. Amphotericin B equivalent of five actinomycete strains against *M. hiemalis*

As shown in Fig. 5, among the five actinomycetes, HIT18 exhibited the most potent antifungal activity against *M. hiemalis*, with an amphotericin B equivalent of 6.32×10^5 μg/mL against *M. hiemalis.* HIT186 exhibited the second-highest antifungal activity, i.e., 5.15×10^2 μg/mL.

Fig. 6 shows the results of the evaluation of the antifungal activity of the five isolates against *R. stolonifer*.

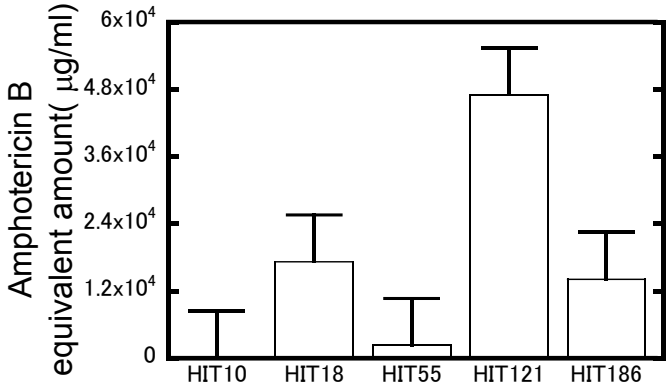

Fig.6. Amphotericin B equivalent of five actinomycete strains against *R. stolonifer*

As shown in Fig. 6, among the five actinomycetes strains, HIT121 exhibited the most potent antifungal activity against *R. stolonifer*. HIT121 exhibited an amphotericin B equivalent of 4.70×10^4 μg/mL against *R. stolonifer*. HIT18 and HIT186 exhibited the second-highest antifungal activity after HIT121, with 1.72×10^4 μg/mL and 2.31×10^4 μg/mL, respectively.

In summary, Fig. 2–6 shows HIT18 exhibited a broad antifungal spectrum against the tested fungi. On the other hand, HIT10 showed potent activity against *Sch. pombe*, and HIT122 was highly active against *R. stolonifer*. As shown in Fig. 6, the antifungal activity of the five isolates against *R. stolonifer* was much stronger than those against the other four fungi.

A survey conducted in a university hospital in India during the decade 1990–1999 showed that fungal infections, especially those caused by *Aspergillus* and *Mucor*, were more common than those caused by *Rhizopus* [13]. Therefore, among the five isolates, we selected strain HIT18, with the most potent antifungal activity against *Aspergillus* and *Mucor* and has a broad antifungal spectrum, as a candidate actinomycete strain that makes a novel antifungal antibiotic. In the future, we plan to purify the antibiotic produced by HIT18 and conduct structural analysis.

IV. Conclusion

Actinomycetes with antifungal activity were screened using plant resources in Hiroshima Prefecture as the source of isolates. As a result, two hundred and eighty-two candidate strains of actinomycetes were isolated and evaluated for antifungal activity against five fungi. The results showed that five strains, HIT 10, HIT 18, HIT 55, HIT 121, and HIT 186, showed antimicrobial activity against all five fungi investigated. The amphotericin B equivalents of these strains were evaluated to determine their relative antibacterial potency. The results showed that HIT 18 exhibited a broad antifungal spectrum against the fungi tested, while HIT 10 was highly active against *Sch. pombe* HIT 122 was highly active against *R. stolonifer*.

Aspergillus and *Mucor* infections are known as typical fungal infections. Therefore, we selected HIT 18 as a promising candidate strain with novel antifungal activity because HIT 18 shows strong antifungal activity against *Aspergillus* and *Mucor* and a broad antifungal spectrum. In the future, we plan to conduct a structural analysis of the antibiotic produced by this strain.

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