Original Article

In vitro antifungal activities of *Actinomyces* species isolated from soil samples against *Trichophyton mentagrophytes*

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Abstract

Background and Purpose: Cutaneous infections arise from a homogeneous group of keratinophilic fungi, known as dermatophytes. Since these pathogenic dermatophytes are eukaryotes in nature, use of chemical antifungal agents for treatment may affect the host tissue cells. In this study, we aimed to evaluate the antifungal activity of *Actinomyces* species against *Trichophyton mentagrophytes* (abbreviated as *T. mentagrophytes*). The isolates were obtained from soil samples and identified by polymerase chain reaction (PCR) technique.

Material and Methods: In total, 100 strains of *Actinomyces* species were isolated from soil samples in order to determine their antagonistic activities against *T. mentagrophytes* in Kerman, Iran. The electron microscopic study of these isolates was performed, based on the physiological properties of these antagonists (e.g., lipase, amylase, protease and chitinase), using relevant protocols. The isolates were identified using gene 16S rDNA via PCR technique.

Results: *Streptomyces flavogriseus*, *Streptomyces zaomyceticus* strain xsd08149 and *Streptomyces rochei* were isolated and exhibited the most significant antagonistic activities against *T. mentagrophytes*. Images were obtained by an electron microscope and some spores, mycelia and morphology of spore chains were identified. Molecular, morphological and biochemical characteristics of these isolates were studied, using the internal 16S rDNA gene. Active isolates of *Streptomyces* sequence were compared to GenBank sequences. According to nucleotide analysis, isolate D5 had maximum similarity to *Streptomyces flavogriseus* (99%).

Conclusion: The findings of this study showed that *Streptomyces* isolates from soil samples could exert antifungal effects on *T. mentagrophytes*.

Keywords: *Actinomyces*, Antifungal agents, *Trichophyton*, DNA/Ribosomal

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Introduction

Numerous *Actinomyces* species, particularly those belonging to the genus *Streptomyces*, are known as antifungal biocontrol agents, inhibiting several fungal plant pathogens [1, 2]. The antagonistic activity of *Streptomyces* species against fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes [3-5]. Chitinase and β-1,3-glucanase are major hydrolytic enzymes in the lysis of fungal cell walls, e.g., the cell walls of *Fusarium oxysporum*, *Sclerotinia minor* and *Sclerotium rolfsii* [6].

In previous research, the antifungal potential of extracellular metabolites from *Streptomyces* species against several fungi has been discussed [1, 2]. However, there is limited information on the antagonistic ability of extracellular metabolites from *Streptomyces* strains to suppress the growth of fungal pathogens, e.g., *Colletotrichum gloeosporioides* and *Sclerotinia rolfsii* (with a broad host range) [2].

Dermatophytes are able to invade keratinized tissues, e.g., the skin, hair and nails of humans and other animals, and cause dermatophytosis,

also known as ringworm or tinea. *Trichophyton mentagrophytes* (abbreviated as *T. mentagrophytes*) is regarded as a complex species and one of the major pathogens causing dermatophytosis [7-9].

Keratin hydrolysis by proteinases (keratinases) is probably an important aspect of fungal pathogenesis. Moreover, some extracellular proteinases have been purified from *Trichophyton* species [10]. Dermatophytes are in fact eukaryotes and use of chemical antifungal agents for treatment may affect the host tissue cells [11]. Therefore, in the present study, we aimed to determine the antifungal activity of actinomycetes species against *T. mentagrophytes.* The isolates were obtained from soil samples and identified by polymerase chain reaction (PCR) technique.

Material and Methods

Sampling, isolation and culture of actinomycetes

In order to obtain actinomycete isolates, 100 random soil samples were collected from different areas of Kerman, Iran at a depth of 10-20 cm beneath the ground. The samples were air dried and passed through a 0.8 mm mesh. After preparing ten-fold serial dilutions in water (10^{-4}, 10^{-5} and 10^{-6}), they were cultured on Casein Glycerol Agar (CGA) and incubated at 29 °C for 5-7 days. The actinomycete colonies were isolated as pure cultures on CGA from day five onwards. The cultures were maintained at 4 °C prior to application [12, 13].

Genetic analysis of PCR

DNA extraction

The five-day-old bacterial culture on CGA was transferred to a 1.5 ml sterile microtube and centrifuged for 5 min at 7,500 g until bacterial precipitation. After discarding the supernatant, 100 µl of protease buffer was added to the pellet and kept at 95 °C for 10 min. The solution was added to the sediment bacteria, based on the bacterial DNA extraction kit by SinaClon BioScience Co. (Karaj, Iran).

After gently shaking the lysis solution for 10 min at 37 °C, 100 µl of the sample and 400 µl of the lysis solution were mixed and shaken for 15-20 sec for rotation. Afterwards, alcohol (300 µl) was added to the microtube for 20 min at -20 °C. The microtube was centrifuged for 10 min at 12,000 g. Following the removal of the supernatant, the microtube was gently stroke on a paper for 2-3 sec in order to remove the remaining precipitated liquid.

One ml of wash buffer was added to the microtube for 3-5 sec and slowly moved into the rotator. Afterwards, the solution was centrifuged at 12,000 g for 5 min and the supernatant was removed. The microtube was kept dry for 5 min at 65 °C. After applying the PCR technique, for 16S rDNA amplification, a forward primer (5'GGATGAGCCCGCGGCCTA 3'), a reverse primer (5' CGGTGTACAAGGCCCGGGAA-CG3') and PCR master mix were used. The PCR reaction was accomplished by 35 cycles of 1 min denaturation at 94 °C, primer annealing at 63 °C for 1 min, primer extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. Afterwards, 45 µl of the obtained products (including the forward primer) was sent to the Bioneer Corporation (Korea) for sequencing and molecular identification, using 16S rDNA [14].

Molecular identification

The PCR products were sequenced, using the forward primer. The sequences were aligned and the species were identified by using the online Basic Local Alignment Search Tool (BLAST) system, available on the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Determination of Minimum Inhibitory Concentration [MIC]

To determine the MICs, different concentrations of crude extracts from active actinomycete isolates (i.e., 10, 5, 2.5, 1.25 and 0.625 mg/ml) with antagonistic effects were prepared in dimethyl sulfoxide (DMSO). MeOH (1:1 v/v) with a suspension of 10^3 cfu/ml *T. mentagrophytes* conidia was tested and incubated at 29 °C for 12 days. MIC was regarded as the lowest concentration inducing growth inhibition. All bioassays were performed in triplicates and the mean values were calculated [15].

Screening of actinomycetes for extracellular enzyme production

Lipolytic activity was detected using a method proposed by Sierra [16]. The proteinase, amylase and chitinase production was determined, using standard methods [17-19]. The electron microscopic studies were performed on active isolates, according to the relevant protocol [20].

Statistical analysis

All experiments were performed under at least three different conditions. One-way analysis of variance (ANOVA) was applied to assess the statistical significance of results, as well as the standard errors. P-value less than 0.05 was considered statistically significant.

Results

Actinomycete isolates from soil samples

From 10^{-4}-10^{-6} dilutions of soil samples, over 100 actinomycetes were isolated in pure cultures in order to perform further investigations.

Phylogenetic analysis of the nucleotide sequence of 16S rDNA gene in active actinomycete isolates

For this purpose, 16S rDNA sequences were amplified by PCR technique, using specific primers. Based on the nucleotide analysis, Ks8 isolate showed maximum homology to *Streptomyces zaomyceticus* strain xsd08149 (98%), 115 isolate displayed complete homology to *Streptomyces rochei* (100%), and D5 showed maximum similarity to *Streptomyces flavogriseus* (99%).

In vitro antifungal activity of actinomycete isolates

Actinomycete isolates, i.e., D5, 115 and Ks8, were evaluated against the tested dermatophytes, using the disk method. The inhibition zones, where no visible pathogenic growth was observed, were representative of antifungal activity.

MIC determination

As presented in Figure 1, The MIC values of actinomycete isolates, i.e., 115, D5 and Ks8, ranged between 25 and 100 mg/ml.

Enzymatic activity assay

Lipase activity

D5 and 115 isolates exhibited various degrees of lipase activity and were able to hydrolyze Tweens. As the results indicated, a sedimentary halo around the colonies was formed by D5 and 115 actinomycete isolates.

Proteolytic activity

Extracellular protease activities of D5 and 115 isolates were determined, based on their ability to hydrolyze casein, i.e., their ability to produce a visible halo around the colonies.

Amylase activity

D5, 115 and Ks8 isolates were able to break down the starch polymer and form colorless zones around colonies after adding the substrate.

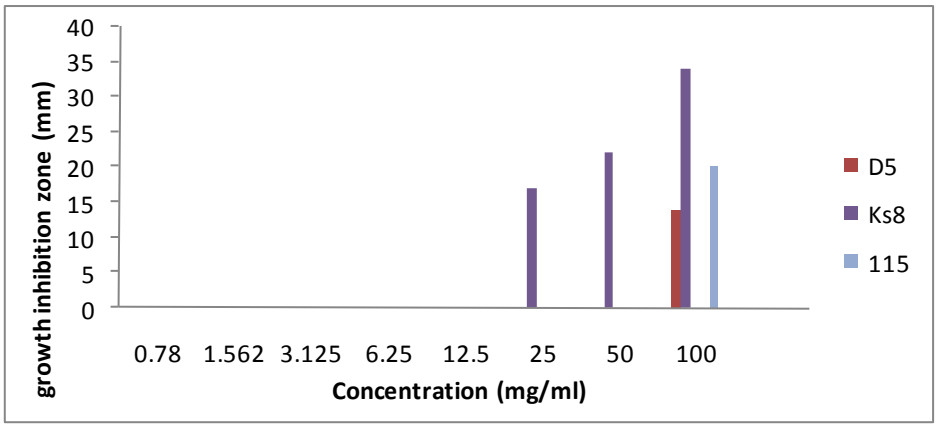

Figure 1. Minimum inhibitory concentrations (MICs) of crude extracts from actinomycete isolates (D5, Ks8 and 115) against the tested dermatophytes, based on the agar well method

Chitinase activity

D5, 115 and Ks8 isolates showed optimal results for chitinase activity. A halo around the colony was detected after growth on the chitin medium.

Electron microscopic studies

Based on the electron micrographs of D5, 115 and Ks8 isolates, all isolates contained long spore chains where the spores had smooth surfaces. Figure 2 shows the spore chains and mycelial morphology of the isolates.

Discussion

According to previous research, 85% of antibiotics, produced from actinomycetes, mainly belong to various *Streptomyces* species [21]. In this study, we aimed to screen and evaluate actinomycete isolates from soil samples in Kerman, Iran in order to determine their antagonist activities against *T. mentagrophytes*, which are the major cause of human dermatophytosis.

In this regard, Deepika et al. (2009) conducted a study on 100 actinomycete isolates from soil samples [22]. Similarly, in the present study, 100 isolates of actinomycete cultures were obtained from different areas of Kerman, followed by in vitro examinations. Actinomycetes were isolated immediately after exhibiting their morphological features and pigmentation within seven days [23].

In the present study, in order to determine the exact antifungal effects of isolates against *T. mentagrophytes*, biological screening was performed, using the agar disk method. D5, 115 and Ks8 isolates exhibited the most significant antagonist activities against *T. mentagrophytes*, considering the formation of inhibition zones.

In a similar study by Shahidi Bonjar et al. (2006), 12 strains out of 130 actinomycete isolates were identified against *Phytophthora drechsleri* [24]. In consistence with the present research, Prapagdee et al. (2008) evaluated 10 actinomycetes isolated from 146 soil samples. As the results indicated, the isolates exerted antifungal effects against three pathogenic fungi [25]. Also, Augustine and colleagues (2005) conducted a similar study, confirming the anti-dermatophytic effects of *Streptomyces rocky* AK39 [26].

Additionally, in a study by Keikha et al. (2015), the most significant antagonistic effects of actinomycete isolates with antifungal activities against *Microsporum canis* isolates, i.e., L1, D5, Km2, Kn1, Ks8 and Ks1, were reported *in vitro* [27]. Based on the MIC evaluation, only three isolates out of more than 100 strains had antifungal compounds against the tested fungi. In the present research, antifungal activities of the isolated strains were demonstrated, indicating the need for further investigations.

Lateral diffusion is a physicochemical approach in which microorganisms are used as indicators of active compounds [28]. In a study by Gross, MIC was estimated at 25-100 mg/ml for active metabolites isolated from the selected strains. Moreover, in a study by Zakir and

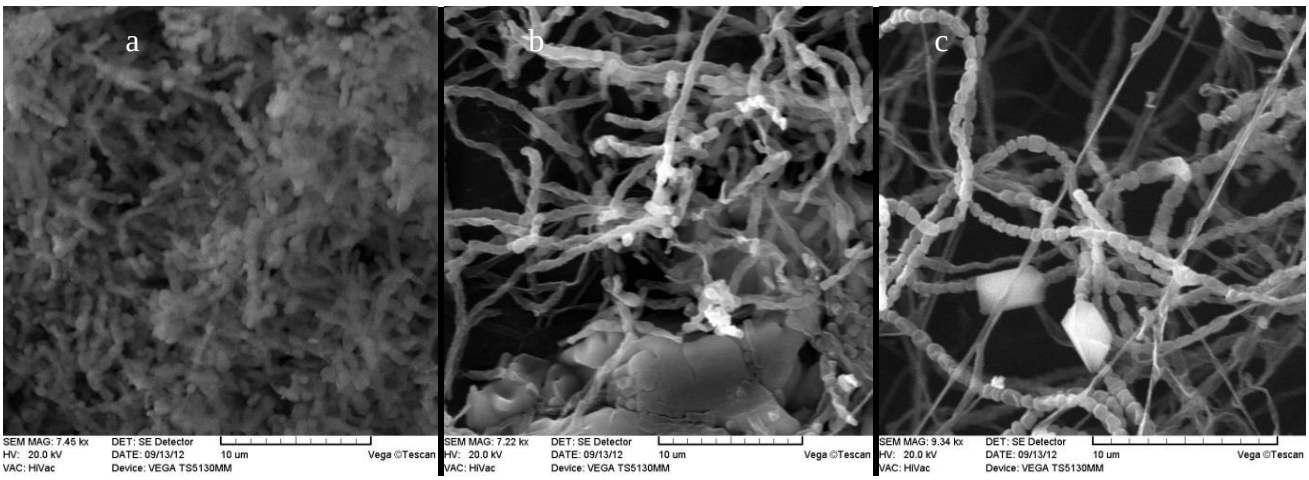

Figure 2. The electron micrographs of actinomycete isolates: (a) 115, (b) D5 and (c) Ks8; all isolates contained spores, mycelia and spore chains.

colleagues (2002), MIC values ranged between 32 and 64 mg/ml for active metabolites isolated from *Streptomyces* species against four Gram-positive and Gram-negative bacteria, respectively [29].

Locci (1989) performed a study in order to identify and classify different *Streptomyces* species, using electron microscopic scanning. The electron microscopic images of actinomycete isolates showed some spores, mycelia and spore chains. Also, according to electron microscopic studies of *Streptomyces* species and *Streptomyces griseus*, the spore chains were spiral, circular and flexible/straight, respectively [20].

The *Streptomyces* lytic activities are mainly induced by lytic enzymes such as chitinase and glucanase [30]. Chitin is a major component of the fungal cell wall and a substrate for chitinase enzymes [31]. In fact, the inhibition of fungi by *Streptomyces* may be related to the production of chitinase [32]. In a study by Baharlouie et al. (2009), 18 actinomycete strains out of 110 isolates showed strong chitinase activity [33]. In this study, D5, 115 and Ks8 isolates showed the best chitinase activities, considering the formation of a halo around the colony after growth on the chitin medium. It should be mentioned that the fungal cell wall contains chitin fibers and the matrix of protein proteases plays a significant role in the degradation of the wall [34].

In a previous study, the role of extracellular proteases in various biocontrol processes was demonstrated in a pathogenic fungus, i.e., *Trichoderma harzianum* [35]. Also, in our study, extracellular protease activities of D5 and 115 isolates were indicated by their ability to hydrolyze casein. D5, 115 and Ks8 isolates were able to break down the starch polymer and form a colorless zone around colonies after adding the substrate.

Conclusion

In this study, we demonstrated that *Streptomycetes* antagonists could exert antifungal effects on *T. mentagrophytes*. The findings of this study could be used for developing new antifungal agents in future. However, various elements should be studied such as short- and long-term side-effects of these agents in animal models, as well as their carcinogenic, teratogenic and environmental effects. Topical evaluations on volunteers should be also considered before making any conclusions.

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Authors' Contributions

All authors were involved in the writing of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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