In Vitro Evaluation of Enzymatic and Antifungal Activities of Soil-Actinomycetes Isolates and Their Molecular Identification by PCR

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1. Background

Human cutaneous infections caused by a homogeneous group of keratinophilic fungi called dermatophytes. These fungi are the most common infectious agents in humans that are free of any population and geographic area. Microsporum canis is a cause of dermatophytosis (Tinea) in recent years in Iran and atypical strain has been isolated in Iran. Its cases occur sporadically due to M. canis transmission from puppies and cats to humans. Since this pathogenic dermatophyte is eukaryotes, chemical treatment with antifungal drugs may also affect host tissue cells.

Objectives: The aim of the current study was to find a new antifungal agent of soil-Actinomycetes from Kerman province against M. canis and Actinomycete isolates were identified by PCR.

Materials and Methods: A number of hundred Actinomycete isolated strains were evaluated from soil of Kerman province, for their antagonistic activity against the M. canis. M. canis of the Persian Type Culture Collection (PTCC) was obtained from the Iranian Research Organization for Science and Technology (IROST). Electron microscope studies of these isolates were performed based on the physiological properties of these antagonists including lipase, amylase, protease and chitinase activities according to the relevant protocols and were identified using gene 16SrDNA.

Results: In this study the most antagonist of Actinomycete isolates with antifungal activity against M. canis isolates of L1, D5, Kst, Kms, Kn, Ks8 and Kst were shown in vitro. Electron microscopic studies showed that some fungal strains form spores, mycelia and spore chain. Nucleotide analysis showed that Ks8 had maximum homology (98%) to Streptomyces zaomyceticus strain xsd08149 and L1 displayed 100% homology to Streptomyces sp. HVG6 using 16SrDNA studies.

Conclusions: Our findings showed that Streptomyces has antifungal effects against M. canis.

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considered to be important hydrolytic enzymes in the lysis of fungal cell walls, as for example, cell walls of 
Fusarium oxysporum, Sclerotinia minor, and S. rolfsii (13). The antifungal potential of extracellular metabolites from 
Streptomycetes against some fungi was previously reported (8, 10). However, data related to the antagonistic ability 
of the extracellular metabolites of Streptomycetes strains to suppress the growth of the fungal pathogens Coleto-
trichum gloeosporioides and S. rolfsii having a broad host range are limited (9).

2. Objectives

This study aims to determine the effect of soil Actino-
mycetes antifungal activity against M. canis isolated from 
soil and identification like Streptomycetes DNA using gene 
16S rDNA.

3. Materials and Methods

3.1. Sampling, Isolation and Culture of Actinomy-
cetes

To obtain Actinomycete isolates over 100 random soil 
samples from different areas of Kerman Province (Sangi 
Park, Sahebazzaman the mosque, Motahari Park and Ne-
shat Park) from depth of 10-20 cm of the ground were 
taken, air dried and passed through 0.8 mm mesh. Ten-
fold serial dilutions prepared in water (10 -4, 10 -5 and 10 
- 6) then cultured on Casein Glycerol Agar (CGA) (casein 
0.3 g/L; KNO3 2.0 g/L; NaCl 2.0 g/L; MgSO4·7H2O 0.05 g/L; 
CaCO3 0.02 g/L; FeSO4·H2O 0.01 g/L; KH2PO4 2.0 g/L; and 
agar (Merck, Germany)18.0 g/L) and incubated at 29°C for 
5-7 days. From day five on, Actinomycete colonies were iso-
lated in pure cultures on CGA. Full grown cultures kept 
refrigerated before use.

3.2. In Vitro Bioassays to Detect the Antifungal Ac-
tivities

Bioassays were performed using Disk-method tech-
nique. In this method, six mm agar plugs of six-day old 
cultures of Actinomycetes on CGA medium were placed 
on lawn cultures of the pathogen on Potato Dextrose 
Agar (PDA) (Merck, Germany). Plates incubated at 28°C for 
10 days. Actinomycetes with clear inhibition zones 
were recorded as positive and selected for further evalua-
tions. Plain agar plugs were used as controls. All bioas-
says were performed in triplicates and the mean values 
calculated.

3.3. Determination of Minimum Inhibitory Concent-
tration (MIC)

To determine the MIC, concentrations of crude sap of 
each antagonist was prepared as 10, 5, 2.5, 1.25, and 0.625 
mg/mL in DMSO: MeOH (Merck, Germany) (1:1, v/v) with suspension of M. canis conidia and tested in well-method 
technique against the pathogen and incubated at 29°C for 
days. The lowest concentration with growth inhibi-
tion was selected as MIC. All bioassays performed in trip-
llicates and the mean values calculated.

3.4. Production of Extracellular Enzymes

Lipase activity test: Growth media containing 10 g pep-
tone (Merck, Germany), 5 g NaCl and 15 g agar/L was 
prepared and autoclaved. After autoclaving, 10 mg of Tween 
80 was added to the media and mixed gently. Disk plugs 
(6 mm) of six-day old cultures of antagonist were pre-
pared and placed on the media as in disk-method bioas-
say. Hydrolysis of Tween 80 by the bacterium presented 
as distinctive zone around the bacterial plug (14).

3.5. Proteolytic Activity Test

Minimal medium containing one g of sucrose (Merck, 
Germany), three g of casein (Merck, Germany), 2 g of cal-
cium chloride and 15 g of agar/L in distilled water was pre-
pared and autoclaved. 6 mm antagonist disk plugs were 
prepared and placed on the media as in disk-method bio-
assay. Hydrolysis of casein and production of clear zones 
were evaluated positive (15).

3.6. Amylase Activity Test

To 5% water agar, 2% (w/w) starch was added, autoclaved 
and poured in Petri dishes. After cooling, 6 mm antago-
nist disk plugs were prepared and placed on the media 
as in disk-method bioassay. Hydrolysis of starch and pro-
duction of clear zones were evaluated positive (16).

3.7. Chitinase Activity Test

Nutrient agar was supplemented with 4% (w/w) colloidal 
chitin (Sigma), autoclaved and poured in Petri dishes. After cooling, 6 mm antago-
nist disk plugs were prepared and placed on the media 
as in disk-method bioassay. Hydrolysis of colloidal chitin and production of clear zones 
were evaluated positive. In this media, chitin was used as the sole carbon source and, therefore, strains with 
chitinase activity, hydrolysed chitin and transparent 
halo around their colonies were evaluated positive and Electron microscope studies of active isolates were per-
formed according to the relevant protocol (17).

3.8. Genetic Analysis of PCR

Extraction of bacterial DNA: From 5 day old bacterial 
culture, 1.5 mL transferred to sterile microtube and cen-
trifuged for 5 minutes at 7500 g till bacteria precipitat-
ed. The supernatant discarded, the pellet received 100 μL 
protease buffer and kept at 95°C for 10 minutes solution 
was added to sediment bacteria according to the kit of 
Bacterial DNA extraction Owned by Sina Colon Iran. The 
lysis solution was given a gentle shake for 10 minutes at 
37°C. 100 μL of the sample with 400 μL lysis solution 
were mixed and shake for 15 - 20 seconds to be rotated.
Then 300 μL of alcohol was added to microtube for 20 minutes at -20°C. The microtube was centrifuged for 10 minutes at 12000 g. The supernatant was discarded and the microtube gently was stroke on a paper for 2-3 seconds to remove the remaining precipitated liquid. One mL of wash buffer added to microtube for 3 - 5 seconds and slowly moved into rotator. It was then centrifuged at 12000 g for 5 minutes and the supernatant was removed. The microtube was kept to dry for 5 minutes at 65°C. After PCR, 45 μL of the PCR products including of the forward primer and molecular identification based on 16SrDNA was sent to the Bioneer Company for sequencing (18). Molecular identification: Sequences were identified using BLAST from National center for Biotechnology Gene Sequences in the database (http:www.ncbi.nlm.gov/BLAST/). This molecular method for identification of sequenced isolates using the 16SrDNA was found at the species level.

4. Results

4.1. Actinomycetes Isolated From Soil

From 10^-4 - 10^-6 dilutions of soil samples, over 100 Actinomycetes isolated in pure cultures on which further investigations performed. In vitro antifungal activity of Actinomycete isolates: Bioassay result of Actinomycete isolates Ks8 and L1 against the tested dermatophyte evaluated in disk-method technique. The inhibition zones in which no visible growth of the pathogen observed are representative of antifungal activity.

4.1.1. Determination of Minimum Inhibitory Concentration (MIC)

As shown in the Figure 1, The MIC for both Actinomycete isolates of L1 and Ks8 isolates determined as 25 mg/mL.

4.2. Enzymatic Bioassays

4.2.1. Lipase Activity

L1 isolate had varying degrees of lipase activity and could hydrolyze tweens. Sedimentary halo formation around colonies was produced by the L1 isolate of Actinomycete.

4.2.2. Proteolytic Activity

Extracellular protease activity of Ks8 and L1 Actinomycete isolates was indicated by their ability to hydrolyze casein (production of clear halo around colonies).

4.2.3. Amylase Activity

L1 and Ks8 isolates was able to break down the starch polymer and created the colorless zone around the colonies after adding the substrate as shown in the Figure 2.

4.2.4. Chitinase Activity

L1 and Ks8 isolates had the best result for chitinase activity. It showed a halo around the colony after growing on the chitin media.

4.2.5. Electron Microscope Studies

Electro micrographs of both Actinomycete isolates of Ks8 and L1 indicated that both isolates bear long spore chains in which spores had smooth surfaces. Figure 3 shows the spore chains and mycelial morphology of both isolates. Phylogenetic analysis of the nucleotide sequence of the 16SrDNA gene of active Actinomycete isolates: 16SrDNA sequences using specific primers was amplified by PCR technique (Figures 4 and 5). Comparison of 16SrDNA sequences active Actinomycete isolates with sequences available in Gen Bank/DDBJ/EMBL using Blast. Nucleotide analysis showed that isolate Ks8 had maximum homology (98%) to Streptomyces zaomyceticus strain xsd08149 and L1 displayed 100% homology to Streptomyces sp. HVG6 (Figure 6).
Figure 3. Electron micrographs of Actinomycete isolates Ks8 (A) and L1 (B). Both isolates bear long spore chains in which spores have smooth surfaces.

Figure 4. Histogram of Ks8 sequence active Actinomycete isolate.

Figure 5. Histogram of L1 sequence active Actinomycete isolate.
5. Discussion

It has been demonstrated that 85 percent of antibiotics which produced and used from Actinomycetes are mainly from various species of Streptomyces (19). In this study, we tried to screen and evaluate Actinomycetes isolated from the soil of Kerman Province for their antagonist activity against M. canis the causative agent of human dermatophytosis. Over 100 isolate of Actinomycetes cultures were isolated from different areas of Kerman’s soil and then the in vitro tests were performed. Actinomycetes were isolated when their morphological features and their pigmentation revealed after seven days (20). Lakshmipathy and Kannabiran (2009) introduced 100 isolates of Actinomycetes isolated from soil (21). In an initial screening to find appropriate antifungal effect of isolates against M. canis, biological screenings performed by agar disk method. The isolates of L1, D5, Ks10, Km2, Kn10, Ks8 and Ks1 showed highest antagonist activity against M. canis based on production of zones of inhibition. In a similar study by Shahidi Bonjar, et al. (2006) they identified twelve strains out of 130 isolates of Actinomycetes against Phytophthora drechsler (22). In another study in Bangkok (2008) similar to what we did; 10 Actinomycetes isolated from 146 soil samples showed antifungal effect against the three pathogenic fungi (23). Augustine and colleagues (2005) also conducted a similar study that proved Streptomyces rocky AK39 has anti-dermatophyte effect (24). Based on their MIC studies they demonstrated only three isolates from more than one hundred strains showed to have antifungal compounds against the tested fungi. In this research antifungal activity of the isolated strains was demonstrated and it showed the importance and potential for further investigation. Lateral diffusion is a physicochemical approach in which microorganisms using it as indicators of active compounds (25). The study conducted by Zakir and colleagues (2002) developed a MIC between 32 - 64 mg/mL for the active metabolite isolated from Streptomyces species against four Gram positive and Gram-negative bacteria respectively (26). As isolates evaluated in this study able to control fungi at a very low concentration then it can be concluded that the antagonistic effects are well studied in the control of microorganisms.

The SEM images obtained from isolates of Actinomycetes showed some form spores, mycelia and spore chains as their morphology revealed. Research in order to identify and classify different species of Streptomyces and electron-type levels of spores in Streptomyces species were reported straight. Also the form of spore chains using electron microscopic studies of Streptomyces species, and Streptomyces griseus was a spiral, circular and flexible straight up (27). Streptomyces lytic activities are mainly the result of lyse enzymes such as chitinase and glucanase (28). Chitin is a major component of the fungal cell wall and as substrate for chitinase enzyme (29). The inhibition of fungi by Streptomyces may be related to the production of chitinase (30). In a study by Bahrhouli et al. (2009) they reported 18 Actinomycete strains out of 110 isolates had strong chitinase activity (31). Since the fungal cell wall contains chitin fibers and the matrix of proteins proteases play a significant role in the degradation of the wall (32). The role of extracellular proteases in various biocontrol processes also was shown in pathogenic fungus Trichoderma harzianum (33). In this research we demonstrated that Streptomycetes antagonists had antifungal effects over M. canis. The outcome of this study can be used for making new antifungal drugs in the future. In this case many various elements should be studied such as evaluation on animal's models for short and long term side effects, carcinogenic and teratogenic effects and environmental impacts. The topical evaluations on volunteers also should be considered before any real conclusion.
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