World Research Journal of Biotechnology
ISSN: 2322-0600 & E-ISSN: 2322-0619, Volume 1, Issue 1, 2013, pp.-04-06.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000297

ISOLATION, MOLECULAR IDENTIFICATION AND EVALUATION OF ANTIFUNGAL ACTIVITY OF SOIL STREPTOMYCES AGAINST DERMATOPHYTES

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Received: July 02, 2012; Accepted: July 11, 2013

Abstract- The aim of this study was to screen the soil's *streptomyces* sp. in Gorgan region in Iran and evaluation of antifungal activity of these bacteria against *Trichophyton rubrum*. A total of 25 strain of *streptomyces* isolates from different soil samples were identify and screened for their antifungal activity by well diffusion method. All isolates are being typed using biochemical and morphological characteristics. Ten isolates had antidermatophytic effects and two of them showed highest antagonistic activity to dermatophyte, these isolates identified by molecular method using PCR and 16s rRNA gene sequence analysis. Genetic analysis confirmed that antagonistic isolates close relationship with known streptomyces species with 99-100% sequence identity. *Streptomyces sp.* SXY37 showed highest antifungal activity against *T. rubrum* by well diffusion method with 23.6 mm zone of inhibition.

Keywords- *Streptomyces*, antifungal activity, soil, dermatophytes

Introduction

Soil bacteria, particularly *Streptomyces* species, produce several biologically active metabolites with antimicrobial and antifungal properties. They also importante for anitbiotics, vitamins and enzymes production and are certain to have a significant role in future biotechnology [1]. Some species of Strepmyces are causative agents of important human and animal diseases, plant pathogens and the rest are involved in the turnover of organic matter [1]. The genus Streptomycete belong to the family Streptomycetaceae in Actinomycetales that grow as mycelial filaments in soil. Because more than 60-75% of known antibiotics that use in human and veterinary medicine are produced by streptomyces species, these bacteria hold a positive of conciderable medical and biological importance. These bacteria are quite commonly found in soil, water and other environment [2].

Dermatophytes are a major group of closely pathogenic fungi that infect skin, hair and nails in humans and animals. These fungi usually do not invade living tissues, but colonize the outer layer of the skin, their products such as acid proteinases, elastase, keratinases, and other proteinases act as virulence factors. Infections caused by dermatophytes commonly referred to as ringworm that transmitted by direct contact with infected host or by direct or indirect contact with infected specimens [3,4]. There is evidence that the dermatophytes have acquired resistance to certain antimycotic drugs and spent worldwide over 500 million dollar for drug against dermatophytosis per year [3,5]. These facts and toxicity of some antifungal drugs has led to the search of alternative compounds, today there is an urgent need to new, safe, nontoxic and more effective antidermatophytic compounds [6-8].

In this study, soil samples were collected from different sites in the Gorgan region in order to streptomyces isolation and determination their antifungal activity against dermatophytic fungi.

Materials and Methods

Collection and Preparation of Soil Sample

Eight different Soil sample were collected for the isolation of streptomycets strain from 4 sites in Gorgan region. Soil samples were taken from top 7.5 - 10 cm soil profile after removing approximately 3 cm of soil surface [9]. Two samples from each region were taken with an auger and placed in dry and sterile polyethylene bags and stored at 4°C until use. Soil samples were pre-treated with calcium carbonate to reduce the number of vegetative bacterial cells and allowing streptomyces spores to survive, this method was required for inhibiting unwanted bacteria.

Isolation of Streptomycetes

In order to isolation of these bacteria 0.5g of treated soil samples was suspended in 9.5ml of sterile distilled water or normal saline and was serially diluted untile 10^{-6} dilution level. Then 0.1 ml of the dilutions was spread on actinomycet isolation agar and ISP-2. The plates were incubated at 28°C for 2 weeks [7,10].

Fungal Strains and Culture Conditions

T. rubrum PTCC 5143 culture was obtained from Iranian Research Organization for Science and Technology (IROST). Fungus was cultured and main maintained on SDA (Sabouraud Dextrose Agar) medium to be used.

Antagonistic Activity Assay

Antifungal activity of each strptomyces isolate against dermatophytic fungus was performed with dual culture and agar well diffusion methods using SDA medium. In this method 2 ml spores suspension (10^6 spore/ml) of test organism was spread on the agar surface of the plate, then two equally spaced wells of 6mm daiameter were made in the agar. All plates then incubated for 48-96 h in 37°C and inhibition zone diameter was measured in mm.

Molecular Characterization

In order to characterization of isolates by molecular methods, PCR and 16S rRNA sequencing were carried out. Genomic DNA of pure subcultures of antagonistic isolates was extracted uising Sinagene Microbial DNA isolaton kit (Sinagene Iran) according to the manufacturer,s specificatins. 45 microliter of PCR product with forward primer send to macrogene company of South Korea (www.macrogen.com) for 16S rRNA sequencing. Universal 16S rRNA primers were used (forward primer was AACTGGAGGAAGGTGGGGAT and revers primer was AGGAGGTGATCCAACCGCA). Sequence data was analyzed with Chromas software version 2.33. the 16s rRNA gene sequence were compared to sequence in the public database using basic local alignment search tool (BLAST) on the national center for biotechnology information (NCBI) website(www.ncbi.nlm.nih.gov). Homology of the 16s rRNA sequence of isolate was analyzed by using BLAST program [11,12].

Results and Discussion

Isolation

Morphological studies of isolates revealed that all 25 isolates from rhizosphere soils were gram positive bacteria with powdery colony. Powdery colony appears convex, concave or flat surface, white, gray to pinkish color with microscopic examination filaments long highly branched and nonfragmenting, arial filament with spirali, coils or multiple branching and long chains spors identified as streptomyces [13-15] [Fig-1]. Among 25 strains, only 10 strains showed antagonistic activity against *T. rubrum*. These isolates identified using biochemical testes such as catalase, nitrate reduction, indole and H_2S production, casein, starch and gelatine hydrolysis according to bergy's manual of systematic bacteriology [Table-1].

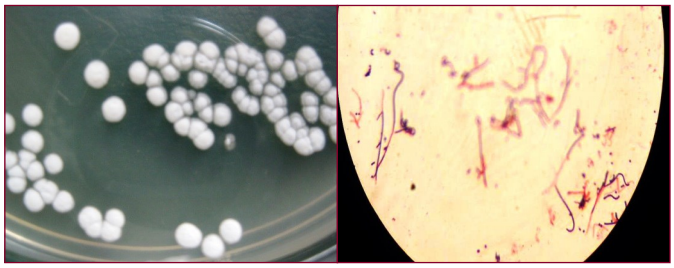


Fig. 1- Morphology of Streptomyces isolates, colony (left) and gram stain (right).

Table1- Biochemical characterization of antagonistic streptomyces isolates

Biochemical characterization	Streptomyces Isolates									
	1	2	3	4	5	6	7	8	9	10
Pigment production	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+
Gram staining	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	+	+	+	+	-	+	+	+
Indol test	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-
Casein hydrolysis	+	-	+	+	+	+	-	+	+	+
StarchCasein hy	+	+	+	+	+	+	+	+	+	+
GelatinCasein hy	+	+	+	+	+	+	+	+	+	+

All of 25 isolates were screened by well diffusion methods and among them 10 isolates showed antagonistic activity against *T. rubrum*. the dual culture revealed that isolates inhibited the growth of *T. rubrum* by well developed inhibition zone [Fig-2] and [Fig-3], [Table-2] and [Table-3].

Antidermatophytic Activity of Isolates

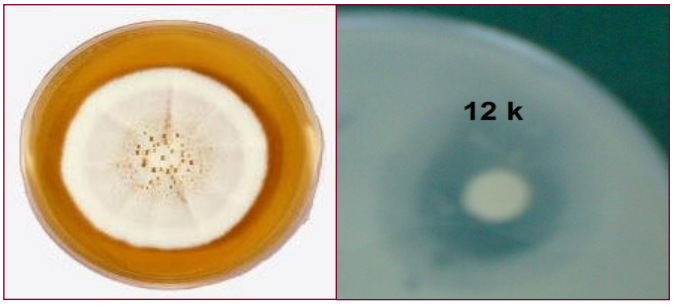


Fig. 2- Morphology of *T. rubrum* on PDA agar (left) and antifungal activity of *Streptomyces sp*.YSEE-1 against *T. rubrum* (right).

Table 2- Antifungal activity of ten isolates of *streptomyces sp*. against *T. rubrum.* (3 readings)

Isolate	Inhibition Zone Diam (MM)									
	1	2	3	4	5	6	7	8	9	10
Code	12 K	14 K	14 J	15 K	16j	16K	17 K	17 J	17p	17x
1	22	18	22	19	20	14	19	17	18	21
2	24	21	26	19	19	17	23	22	17	18
3	23	20	23	20	21	16	21	18	20	20
Mean	23	19.6	23.6	19.3	20	15.6	21	19	18.3	19.6

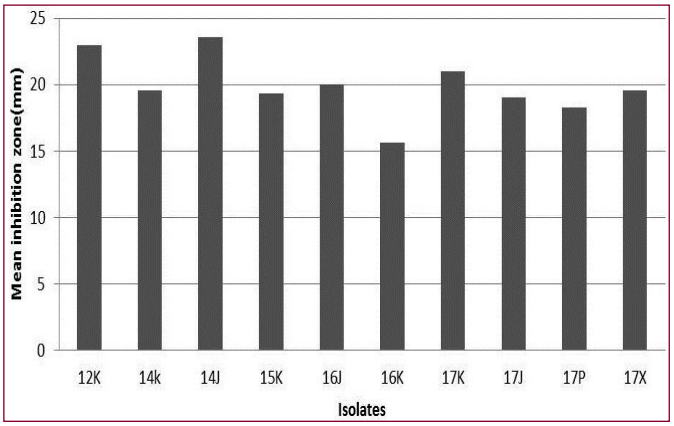


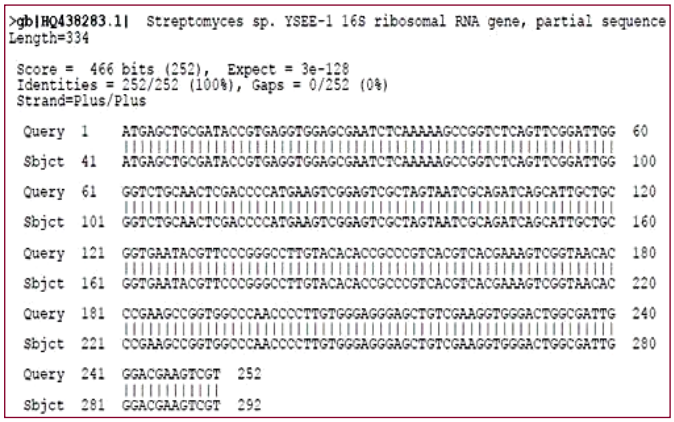
Fig. 3- Antifungal activity of ten isolates of *streptomyces sp*. against *T. rubrum*

Table 3- The mean and standard deviation of Inhibition zones (3 readings)

Test organism	Mean	Std. Deviation
12 K	23	1
14 K	19.6	1.527
14 J	23.6	1.527
15 K	19.3	0.557
16j	20	1
16K	15.6	1.527
17 K	21	2
17 J	19	2.645
17p	18.3	1.527
17X	19.6	1.527

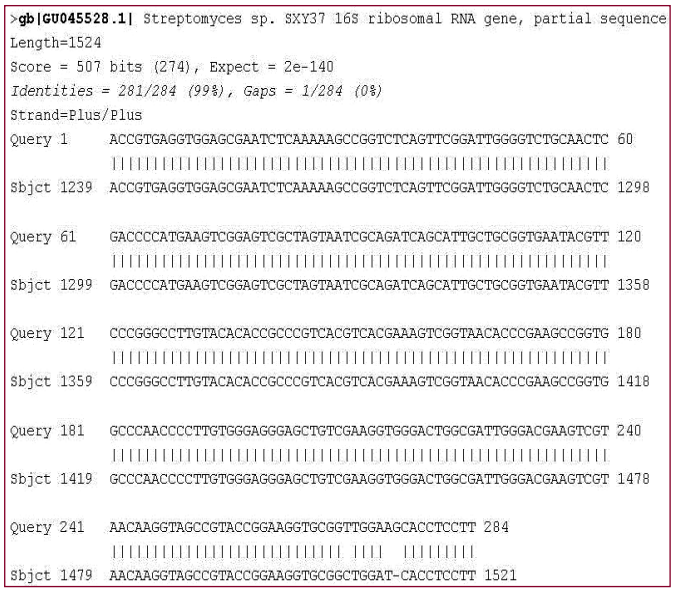
16s rRNA Sequences of the Antagonistic Isolates

Blast analysis of partial 16S rRNA gene sequences showing that the 2 isolates were closely affiliated with genus Streptomyces. The isolates shared sequence identity of between 99-100% with known Streptomyces species. Isolate 1 and 3 having a sequence identity of 100% and 99% with *Streptomyces sp*.YSEE-1 and *Streptomyces sp*. SXY37 respectively [Fig-4] and [Fig-5].



```
>gb|HQ438283.1|  Streptomyces sp. YSEE-1 16S ribosomal RNA gene, partial sequence
Length=334

 Score =  466 bits (252),  Expect = 3e-128
 Identities = 252/252 (100%), Gaps = 0/252 (0%)
 Strand=Plus/Plus

  Query  1    ATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Sbjct  41   ATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGG  100

  Query  61   GGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Sbjct  101  GGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGC  160

  Query  121  GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Sbjct  161  GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC  220

  Query  181  CCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Sbjct  221  CCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTG  280

  Query  241  GGACGAAGTCGT  252
              ||||||||||||
  Sbjct  281  GGACGAAGTCGT  292
```

Fig. 4- 100% homology of K12 isolate (RW primer) with *Strepyomyces sp*. YSEE-1



```
>gb|GU045528.1| Streptomyces sp. SXY37 16S ribosomal RNA gene, partial sequence
Length=1524
Score = 507 bits (274), Expect = 2e-140
Identities = 281/284 (99%), Gaps = 1/284 (0%)
Strand=Plus/Plus
Query 1     ACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTC 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1239  ACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTC 1298

Query 61    GACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1299  GACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT 1358

Query 121   CCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTG 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1359  CCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTG 1418

Query 181   GCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGT 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1419  GCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGT 1478

Query 241   AACAAGGTAGCCGTACCGGAAGGTGCGGTTGGAAGCACCTCCTT 284
            ||||||||||||||||||||||||||||| ||||  |||||||||
Sbjct 1479  AACAAGGTAGCCGTACCGGAAGGTGCGGCTGGAT-CACCTCCTT 1521
```

Fig. 5- 99% homology of 14J isolate (RW primer) with *Strepyomyces sp*. SXY37

Conclusion

Streptomyces are the most common saprophytic bacteria exist in the soil and are known for their potential as antibiotic producer. The result of the present investigation reveal that the *Strepyomyces sp*. YSEE-1 and *sp*. SXY37 isolated from soil show potential antidermatophytic activity against the dermatopyte *T. rubrum*. These isolates might be good candidates to dermatophytic fungi biocontrol.

Acknowledgment

This work was supported by Islamic Azad University, Science and Research Branch, Tehran, Iran. I wish to express my thanks to my supervisor Seyed Jamal Hashemi Hazaveh and other professors in department of mycology especially Mansour Bayat and Mahmoud Jamshidian for their warms encouragement.

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