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CHARACTERIZATION AND ANTAGONISTIC POTENTIAL OF SOIL ACTINOMYCETES AGAINST PATHOGENS OF HUMAN MYCOSIS

Pragya Jadon, Ramendra Singh Parmar, Charu Singh and Ajay Kumar*
Department of Life Sciences, ITM University, Gwalior - 475001, Madhya Pradesh, India.
*Corresponding author Email: **a.kitmmicrobio@gmail.com**
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Abstract: This study was conducted to isolate and identify the biologically potential actinomycetes. Total of 70 cultures were isolated from different habitat of Gwalior region, Madhya Pradesh, India. All isolated actinomycetes evaluated for their ability to inhibit human pathogens, *i.e. Microsporum canis*, *Microsporum gypseum, Microsporum fulvum, Tricophyton rubrum* and *Tricophyton mentagrophyte*. All isolates were screened for their antifungal activity by agar well method against dermatophytes. After screening out of these isolates, only one isolate designated as AP-27 showed antifungal activity against *Microsporum canis*, *Microsporum gypseum,* and *Tricophyton rubrum.* Cultural, morphological and biochemical studies of isolate AP-27 classified it into *Streptomyces* spp. The 16S rRNA gene sequence of isolate AP-27 classified as *Streptomyces griseus*. This study proves that actinomycetes isolated from soil of Gwalior region have good antifungal activity against dermatophytes.
Keywords: Soil actinomycetes; Antifungal activity; Dermatophytic fungi; secondary metabolite.
Postal Address: Dr. Ajay Kumar, Assistant Professor, Department of life sciences, ITM University, Turari Campus Jhansi Road Gwalior- 475001, Madhya Pradesh, India, Contact No.- +91 9074213612.

INTRODUCTION

Actinomycetes are Gram-positive bacteria with high G+C content showing filamentous growth like fungi. They are aerobic and have diversity in ecological habitats such as soil, fresh water, sewage and marine water. Actinomycetes are producer of theruptically used compound and widely used antibiotics. Production of secondary metabolite is an important characteristic feature of actinomycetes. Many antibiotics like streptomycin, gentamycin, rifamycin, erythromycin and many other well-known anticancer drugs are the product of secondary metabolite by actinomycetes (Valan *et al*., 2012). Many other commercially used compounds like antibiotics, antiparasitic, antifungal agents, anticancer and immunosuppressive agents are produced by actinomycetes (Bundale *et al.,* 2015). About 20% to 25% world's population have the problem of Dermatomycosis commonly known as ringworm infection. It is an infection of skin, hair and nails. Keratinized tissues are the main target of dermatophytic fungi. Three genera of dermatophytes known as *Microsporum, Tricophyton*, and *Epidermatophyton* are responsible of all dermatophytic infection in human. Opportunistic infection becomes a big problem in the immunocompromised host, so new, safe and more effective compounds are focus of research. Antibiotic resistance to pathogenic fungi is also becomes a big problem. Because of human mycoses dermatophytes get attention of medical epidemiologists. Many antifungal agents like azole, allylamine etc. are used to manage these infection but many drawbacks becomes a serious problem like drug resistance and severe side effects (El Gendy *et al.,* 2016).

EXPERIMENTAL

Sample collection

Soil samples were collected from the different habitat of Gwalior region, Madhya Pradesh,

India. These sites were Playground soil, rhizopheric soil of medicinal plants, Agricultural field soil, Poultry farm soil, Industrial waste and Sewage soil. Soil samples were collected at the depth of 5-10 cm, top surface layer of soil was removed and central portion of soil was collected in sterile plastic bags (Gunasekaran *et al.,* 2013). All samples were labelled and kept in the BOD incubator at 4^{O}C for further use.

Isolation of Actinomycetes

Isolation of actinomycetes was done by the serial dilution and pour plate technique. For isolation of actinomycetes different media were used like Starch casein agar, Glycerol Asparagine agar, Actinomycetes isolation agar, Soil extract agar, Yeast extract malt extract agar ,Starch nitrate agar, Inorganic salt starch agar and Oat meal agar . All plates were incubated at 30^{O}c for 7 to 21 days. After incubation isolated colonies were purified on respective fresh media and stored at 4^{O}C until further use (Alimuddin *et al*., 2011)

Isolation and Identification of Test Dermatophytes

Dermatophytes used as test organisms like were isolated originally from hospital waste land and drainage soil, collected from hospitals of Gwalior region. Isolation of dermatophytes was done by hair-bait technique (Shukia *et al*., 2003). 50g of each soil sample placed into sterile petri dish and baited with sterilized hair piece. Soil was moistened with 5-10 ml of sterilized distilled water and incubated at room temperature for 3-4 weeks. The fungus which appeared on baits were transferred on Sabouraud's dextrose agar supplemented with antibiotics *i.e.* cyclohexamide and tetracycline. Plates were incubated at 28^{O}C for 10 -21 days. Isolated cultures were identified by morphology, culture characteristics and microscopic examination (Frey *et al.,* 1886; Rippon *et al.,* 1988; Laron, 1995; Ekwealor *al*., 2015; El Gendy *et al*., 2016). Identified fungal culture were then transferred into slant of SDA and kept at 4^{O}C for further use.

Screening of Soil Actinomycetes

All isolated actinomycetes were screened for their antifungal activity against dermatophytes. Screening was done by primary and secondary method. Primary screening was done by double layer method. In this method starch casein agar medium was prepared and thin layer was poured into the plate then actinomycetes were inoculated by spot inoculation method in the centre of medium. plates were incubated for 3 to 4 days at 30^{O}C then second layer of SDA was poured on same plate and culture of dermatophytes were spread by spread plate method, plates were incubated for 6-8 days at 28^{O} C and after incubation zone of inhibition was measured (Ayari *et al.,* 2012). Secondary screening was performed by agar well diffusion method. Two media were prepared i.e. Sabourauod's dextrose agar and Starch casein broth. Most promising isolate was inoculated into starch casein broth and incubated in a rotary shaker under agitation at 30^{O}C ±1 for 5-7 days at 200 rpm. The fermented broth was centrifuged at 10,000 rpm for 10 min, filtered through a Whitman No.1filter paper. The mycelium free culture filtrate was tested for antifungal activity (Kannan *et al.,* 2013). Two wells were made on Sabouraud dextrose agar plate by well cutter and broth culture of tested fungal pathogens were spread by spread plate technique, then wells were loaded with Starch casein broth (150 µl) and one with cyclohexamide as a positive control. All plates were incubated at 28^{O}C and zone of inhibition was measured after 7 days of incubation (Pandey *et al.,* 2011).

Cultural and Morphological Characteristics of Isolate AP-27

Cultural characteristics of isolate AP-27 such as colour of aerial mycelium, colour of substrate mycelium, and pigmentation were studied on different media like Starch casein agar, Tryptone-yeast agar, Maltose Yeast extract agar, Potato Dextrose Agar, Nutrient Agar, Czapex dox agar, Starch agar medium and Sabouraud dextrose agar To study the morphological characteristics Gram staining was done and spore structure was examined by electron microscopy (Scanning electron microscope) (Khamna *et al*., 2009).

Biochemical characteristic

After screening, promising isolate was selected for Biochemical testing. Biochemical test used were hydrolysis of gelatin, Casein hydrolysis,

Methyl red and Voges Proskauer Test, Nitrate reduction, Hydrogen sulfide production and Fermentation of carbohydrates like - Glucose Fructose, Sucrose, Ribose, Galactose, Maltose, Xylose, Rahaminose, Raffinose and their acid gas production was also observed (Singh *et al*., 2016).

Identification of isolate AP-27 by 16S rRNA Sequencing

The selected isolate AP-27 was subjected to molecular characterization and phylogenetic analysis. Firstly genomic DNA was isolated, extracted and amplified by using PCR. PCR product was sequenced by using universal primers (forward primer -5'-GCCTAACACATGCTGG-3' and reverse primer -3'- GTATTACCGCGGCTGCTGG-5'). Molecular Evolution Genetics Analysis (MEGA) software version 7 was used to carry out phylogenetic analysis of the alignment (Isik *et al.,* 2014).

RESULTS AND DISCUSSION

Isolation of Actinomycetes

Total of 70 soil actinomycetes cultures were isolated from different soil samples of Gwalior region. It was shown that maximum number of isolates was obtained from rhizospheric soil of medicinal plants following by agricultural field soil (Figure 1). Among the different media used for isolation Starch casein agar media was proved to be the best media, maximum no. of isolates were isolated with this media as compare to other media (Figure 2). All isolated cultures were grouped based on different color groups of aerial mycelium, substrate mycelium and soluble pigment.

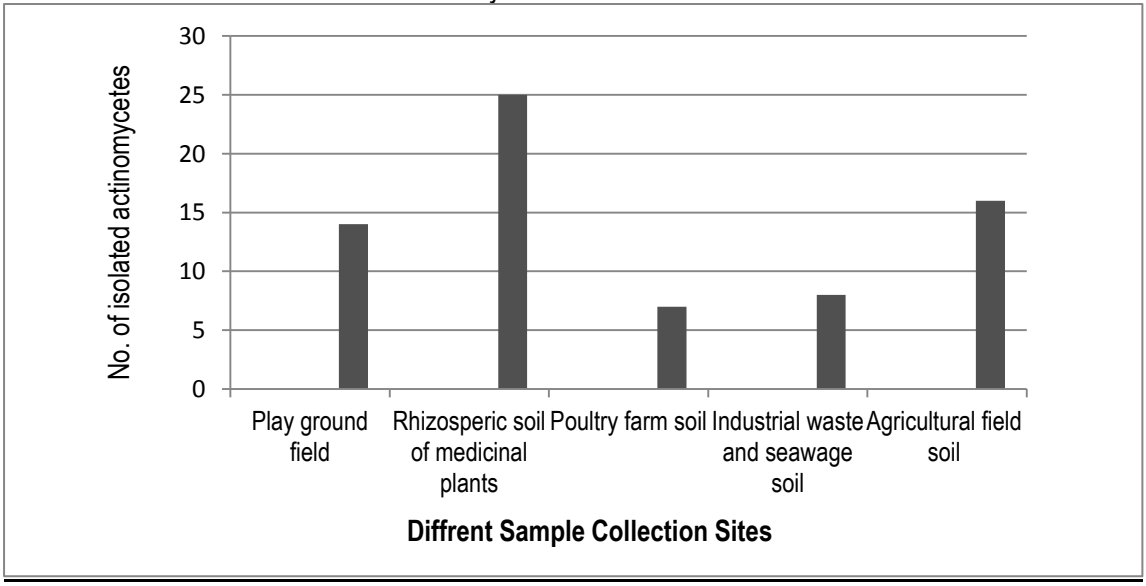

Figure 1. No. of isolated actinomycetes from different soil sample collection sites of Gwalior region

Table 1. Morphological and Microscopic Characteristics of isolated Dermatophytes

Isolate	Colony appearance	Pigment production	Microscopic study	Isolate Identified
Pd1	White to dark cream	Light yellow to brown	Macroconidia produced with spherical shaped thick wall	*M. canis*
Pd2	Cream to dark brown colored	Yellow brown pigment	Macroconidia with spiny thin wall	*M. gypseum*
Pd3	Light brown surface with white border	Dark red surface	Longer bullet shaped Macroconidia. Some clavate Microconidia produced	*M. fulvum*
Pd4	White colored fluffy and sticky	Deep red wine colored.	Small Pyriform Microconidia were present. Macroconidia not seen	*T. ruburum*
Pd5	White powdery surface	Yellow to light brown	Thin smooth walled Microconidia were present	*T. mentagrophyte*

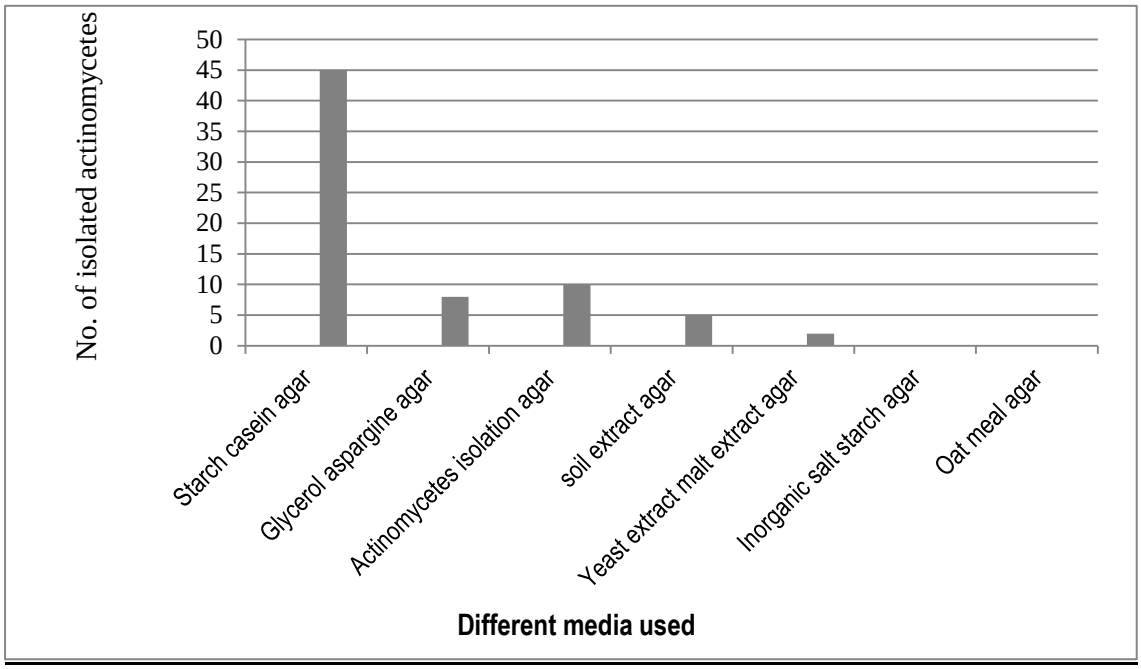

Figure 2. Preference of Media for Isolation of Soil Actinomycetes

Isolation and Identification of Dermatophytes

Total of 5 fungal cultures were identified as keratinophilic or dermatophilic fungi they were *Microsporum canis*, *Microsporum gypseum, Microsporum fulvum, Tricophyton ruburum* and *Tricophyton mentagrophyte*. Identification was done on the basis of morphological, cultural and microscopic examination and it was found that all dermatophytic fungi shown different morphological features like colony appearance and colony colour (Table 1). *M. canis* and *M. gypseum* shown long incubation period from 7-10 days as compare to other fungi.

Screening of soil Actinomycetes

Among the 70 isolates, 16 actinomycetes showed antifungal activity against *M.canis, M.gypseum* and *T.ruburum* in primary screening. In secondary screening 6 actinomycetes were active against *M.canis, M.gypseum* and *T.rubrum.* Among these 6 isolates one isolate was selected as a most promising isolate designated as AP-27 obtained from rhizopheric soil of medicinal plants. This isolate was selected for further studies on the basis of maximum zone of inhibition (0- 22 mm) against maximum no. of dermatophytes. The Zone of inhibition by isolates is shown in Table 2.

Table 2. Antifungal Activity of isolates against Dermatophytes

	Zone of inhibition (mm)		
Isolates	***M.canis***	***T.ruburum***	***M.gypseum***
AP-7	22	-	20
AP-10	-	14	12
AP-19	12	-	12
AP-27	26	20	22
AP-35	16	16	-
AP-49	8	12	-

Cultural, Morphological and Biochemical characteristics of isolate AP-27

Morphological and cultural analysis of isolate AP-27 on Starch casein agar media suggested that isolate produced white colored spore mass and light yellow colored substrate mycelium. Isolate AP-27 produced pink colored pigment soluble in media. Light microscopy of isolate AP-27 was observed and concluded as gram positive and dichotomously branched spore chain (Figure 3). Observation under scanning electron microscope (SEM) shown spiral spore chain, terminal of spores was open and surface was smooth (Figure 4). All morphological and cultural characteristics of isolate AP-27 presented in Table 3. Result presented in Table 4 showed that isolate shown maximum and fast growth on Starch casein agar media as compare to other media, pigment production was also varied with

change in media like on SCA media isolate produced light pink colored pigment while colour of pigment was dark pink with starch agar medium. Biochemical study of isolate AP-27 suggested that isolate had the ability to degrade casein and starch and isolate also shown positive results for Simmon citrate and MRVP test while H_2S and nitrate reduction was not observed (Table 5). Many carbon sources was utilized by isolate AP-27 like Glucose, Fructose, Sucrose, Ribose, Maltose, Xylose while Rhamnose and Raffinose were not used, results are presented in Table 6. From cultural, morphological and biochemical characterization of isolate AP-27, it was concluded that isolate belongs to *Streptomyces* genus.

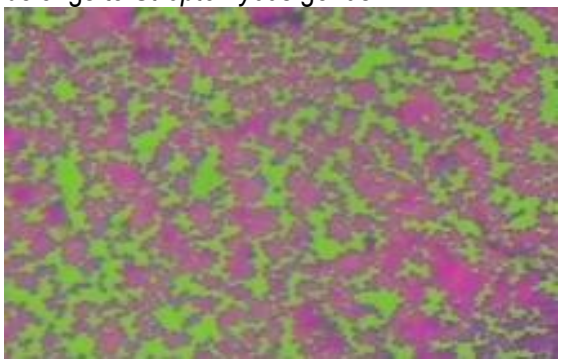

Figure 3. isolate AP-27 under x100 Resolution

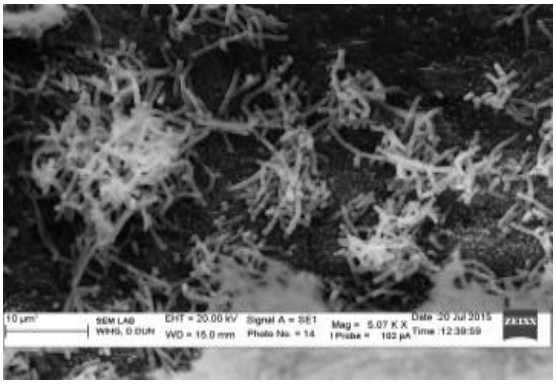

Figure 4. Scanning Electron micrograph of isolate AP-27

Table 3. Morphological and Culture characteristics of Isolate AP-27

Properties	Characteristics of AP-27
Gram's staining	Gram positive
Hyphae	Present
Mycelium	Present
Color of aerial mycelium	White
Color of substrate	Light yellow
Spore mass colour	White
Pigment production	Light pink (soluble in media)
Spore morphology	Spiral (open)
Spore surface	Smooth

Table 4. Culture characteristics of isolate AP-27 on different media

Media	Growth	Aerial mycelium colour	Substrate mycelium colour	Pigment production
Starch casein agar	Excellent	White	Yellow	Light pink
Tryptone-yeast agar	-	-	-	-
Maltose Yeast extract agar	-	-	-	-
Potato Dextrose Agar	Average	White	Yellow	Light Pink
Nutrient Agar	-	-	-	-
Czapex dox agar	-	-	-	-
Starch agar medium	Good	White	Light Yellow	Dark pink
Sabouraud dextrose agar	Good	white	Light Yellow	Light Pink

Table 5. Biochemical characteristics of isolate AP-27

Biochemical Characteristics	Isolate AP-27
Casein hydrolysis	+ve
Simmon citrate	+ve
Methyl red	+ve
Voges Proskauer	+ve
Nitrate reduction	-ve
H_2Sproduction	-ve
Starch hydrolysis	+ve

Table 6. Utilization of different carbon sources by isolate AP-27

Carbohydrate Test	AG formation
Glucose	Acid gas
Fructose	Acid gas
Sucrose	Gas
Ribose	Gas
Galactose	Acid gas
Maltose	Acid gas
Xylose	Gas
Rhamnose	Gas
Raffinose	Gas

Identification of isolate AP-27 by 16S rRNA Sequencing

Molecular characterization suggested that 16S rRNA sequence of isolate AP-27 had 98% sequence identity with 16S rRNA gene sequence from several *Streptomyces sp*. This result clearly suggests that the isolate AP-27 belongs to the genus *Streptomyces* spp. Phylogenetic analysis shown that isolate AP-27 closely related to *Streptomyces* sp. and showed high similarity towards *Streptomyces griseus* (Figure 5). Valan *et al*., (2012) concluded in their research that many effective antifungal compounds were produced by group of actinomycetes. Many species of actinomycetes have the ability to inhibit the growth of various fungus (Ayari *et al.,* 2012). Very few actinomycetes were reported from Gwalior region Madhya Pradesh with antidermatophytic activity. In the present study 70 actinomycetes were isolated from different areas of Gwalior region. All actinomycetes were screened for their antifungal activity against *M. canis, M. gypseum M. fulvum, T. ruburum and T. mentagrophyte.* Many researchers used different media for isolation of actinomycetes and they find that among different media SCA medium was proved to be the best medium (Gunasekaram *et al*., 2013).

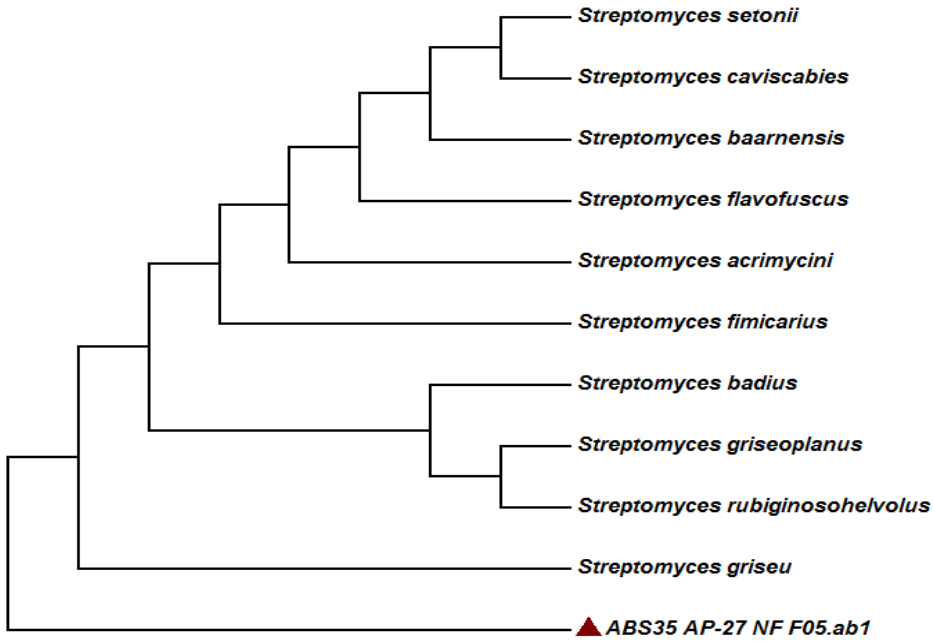

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship of isolate AP-27 with recognized member of the genus Actinomycetes

Total of 6 media were used in this study for isolation, among them SCA media was proved to be most effective for isolation of actinomycetes. In the present study soil actinomycetes were screened for their antifungal activity by Agar well diffusion method. In this method 6 isolates possess antifungal activity against dermatophytes zone of inhibition was measures 0-26mm. The isolate AP-27 was found to be most inhibitive against *M. canis, M. gypseum* and *T. ruburum.* Similarly Valan *et al*., (2012) identified *Streptomyces* sp. active against pathogenic bacteria and dermatophytes from coast of Bay of Bengal. Bharti *et al.,* (2010) screened 94 actinomycetes out of 316 isolated from Gharwal region uttarakhand and found that isolates shown antifungal activity against dermatophytes and other fungal agents. Cultural, morphological and biochemical characteristics of isolate AP-27 suggested this isolate as gram positive with spiral spore chain with smooth surface and classified it into *Streptomyces* spp. Alimuddin *et al.,* (2011) reported in their studies that morphological, cultural and biochemical characteristics play important key role for identification of actinomycetes, they characterized the actinomycetes on the basis of aerial mycelium colour, substrate mycelium colour and soluble

pigment. They find grey colored aerial mycelium and concluded that *Streptomyces* group was dominant. The 16S rRNA gene sequence of isolate AP-27 classified as *Streptomyces griseus.* Many researchers shown that many species of *Streptomyces* produced antibiotics, antiparasitic, antifungal, anticancer and immunosuppressive agents (Bundale *et al.,* 2015). Further investigations are needed in order to obtain detail information about the active metabolites produced by isolate AP-27.

CONCLUSION

This study can be concluded that the isolated actinomycetes showed significant antagonistic activities against dermatophytes and reported first time from soil of Gwalior region. Findings of present study showed that isolation and characterization of naturally occurring actinomycetes from Gwalior region may be useful for identification of new bioactive compound against dermatophytes. From present investigation isolated actinomycetes may be commercially formulated as effective bio-control agents for the management of dermatophytes.

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